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Research Article

Comparison of Anaesthetic Effects of Intravenous Emulsified Isoflurane and Inhaled Isoflurane in Dogs

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Abstract

To compare anaesthetic effects between emulsified isoflurane (EI) through intravenous route and inhaled isoflurane in dogs, 16 healthy adult non-purebred dogs were randomly divided into intravenous anaesthesia groups with 8% EI (EI group) and an induction anaesthesia group (IA group) with vapor isoflurane. The anaesthetic effect was assessed by observing dog's reaction, obvious side effects and baseline physiological parameters, including of Mean Arterial Pressure (MAP), Respiratory Rate (RR), Diastolic Arterial Pressure (DAP), Heart Rate (HR), Systolic Arterial Pressure (SAP), Oxygen saturation (SpO_2) were recorded at 0, 5, 10, 20, 30, 40, 50, 60, 70 and 80 min after administered anaesthetic agents. The results revealed that no conspicuous differences were discovered between the groups during anaesthesia. This study showed that there is a good prospect for using EI intravenously in dog anaesthesia.

Keywords: *Isoflurane, Emulsified isoflurane, Anaesthetic effects, Dog*

Köpeklerde İntravenöz Emülsifiye İzofluran ve İnhale İzofluranın Anestetik Etkilerinin Karşılaştırılması

Öz

İntravenöz yoldan verilen emülsifiye izofluran (EI) ile inhale izofluran arasındaki anestezik etkileri karşılaştırmak için, sağlıklı yetişkin safkan olmayan 16 köpek rastgele %8 EI intravenöz anestezi grubu (EI grup) ve inhale izofluran anestezi grubu (IA grup) olarak iki deneme grubuna ayrıldı. Anestezik etki, köpeğin reaksiyonu, belirgin yan etkiler ve Ortalama Arteriyel Basınç (MAP), Solunum Hızı (RR), Diyastolik Arteriyel Basınç (DAP), Kalp Hızı (HR), Sistolik Arteriyel Basınç (DAS), Sistolik Arteriyel Basınç (SAP), Oksijen doygunluğu ($Sp0_2$), anestezik ajanların uygulanmasından sonraki 0, 5, 10, 20, 30, 40, 50, 60, 70 ve 80. dakikalarda kaydedildi. Sonuçlar, anestezi sırasında gruplar arasında belirgin bir fark bulunmadığını ortaya koydu. They all play a good role in dogs, as time goes on, these parameters are slightly reduced, and there are also physiological acceptable limits. Bu çalışma, köpek anestezisinde intravenöz olarak EI kullanımının iyi bir seçenek oluşturduğunu gösterdi.

Anahtar sözcükler: *Isoflurane, Emülsifiye isoflurane, Anestezik etki, Köpek*

INTRODUCTION

Anaesthesia is currently used for transportation and experimental work in many animal species, especially in surgical operation aspects. Considering economic, technological and even legal constraints, volatile anesthetics, such as, isoflurane, sevoflurane or halothane, cannot be used in some states. Intravenous (I/V) injection of volatile anesthetics can cause organism damage, such as changing hemodynamic variables and pulmonary function [1]. However, several preliminary investigations have confirmed to a certain extent that intravenous injection of emulsified halothane or isoflurane has no harmful effects on animals [2,3]. Over the years, emulsified isoflurane was appeared frequently to our laboratory's (The Key Laboratory of Zoonosis of Liaoning Province, College of Animal Science &Veterinary Medicine, Shenyang Agricultural University) investigations, but it has not been used all over the world, in sharp contrast to inhalational isoflurane. Emulsified isoflurane is a special lipid emulsion state of isoflurane, and one component content of the preparation is 30% Intralipid (Libang Pharmaceutical Co, Ltd., Xi'an, China), a

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bacterium-free, non-pyrogenic axunge emulsion provided for I/V administration. Compared with the traditional route of inhalation, apply for emulsified isoflurane demands for changes in dosage of estimate, but they are handy and fast acting to apply I/V, and also does not need additional volatilizing equipments. So it could be used in the areas with complex natural environments. Recent studies already put forward that emulsified isoflurane emerged good anaesthesia after I/V injection and provided certain advantages [4]. The main objective of this research was to compare anaesthetic effects between intravenous EI and inhaled isoflurane in dogs.

MATERIAL and METHODS

Animals

With the permission of the Animal Care and Use Committee of Shenyang Agricultural University (201604-001), 16 healthy adult non-purebred dogs were applied for this study, included 8 females and 8 males, with body weights 5.2±0.4 kg and ages 15±3 months. The dogs were housed individually and fed the same kinds and equal amounts of dry food twice a day and water adequate and casual for 15 days. Before the formal experiment, basic examination (including biochemical profile, electrocardiography, and complete blood count) was performed. The results stated clearly that all dogs were apparently healthy without significant changes of clinical disease.

Materials

Emulsified isoflurane (EI) was set up on the basis of use an aseptic technique as described before [1,5]. Briefly, taking 0.8 mL liquid isoflurane (Heilongjiang Key Laboratory of Anesthesiology and Intensive Care Research, Harbin, China) and 9.2 mL 30% intralipid (Libang) to a 10 mL glass ampoule, and then, the ampoule was conserved after seal hermetically by an alcohol blowtorch. Shake ampoules with a vibrator forcefully, maintain for 15 min, to dissolve isoflurane into the lipid emulsion. Stored at room temperature for six months, there were no lipid droplets be found in liquid isoflurane, and the concentration of lipid droplets remained unchanged.

Study Design

Organized 16 dogs into the intravenous anaesthesia group with 8% EI (EI group) or the inhalation anaesthesia group with isoflurane vapor randomly (IA group). Before operation apiece begun, fasting with no food for 12 h, and no drinking water for 3 h, intravenous catheter (26 G, Jinhuan Medical Products Co., Ltd., Shanghai, China) was placed on brachiocephalic vein of each dog for 2 h.

Dogs were arranged for a sternal recumbency on a limitative holder. Dogs were put on an obedient respiratory mask which link up together with an anaesthesia machine (Excel 210 SE; Datex-Ohmeda, Madison, USA) contain an isoflurane atomizer and dogs primary breathed 100% oxygen before foggy EI or isoflurane was administered. In EI group, atropine sulfate (0.03 mg/kg, Qilu Animal Health Products Co., Ltd, Jinan, China) was administered by intramuscular injection before anaesthesia, after 15 min every dog recepted 1 mL/kg of emulsified isoflurane in 20 sec by intravenous push, then they were intravenous pushed 8 mL/kg·h^{-1} of EI by intravenous infusion. As soon as intravenous infusion of drugs for anaesthesia, timing notes were jot down. After 60 min of pushing drugs, the intravenous anesthetic was stopped. But recording the time was continued until 80 min. In IA group, atropine sulfate (0.03 mg/kg) was administered by intramuscular injection before anaesthesia, after 15 min each dog received 5 mg/kg of propofol (Libang) by intravenous push, and the dogs were intubated after induction of general anaesthesia. 3% isoflurane was inhaled at fast rate for 3 min through anaesthesia machine. Then 1.5% isoflurane was maintained for 60 min. As soon as inhalation of 3% isoflurane, time notes were recorded again. After 60 min of drugs intervention, inhalation of anesthetic was stopped. But recording the time was continued until 80 min.

Physiological Monitoring

Physiological indicators and anaesthesia grades were assessed at time nodes, which were 0, 5, 10, 20, 30, 40, 50, 60, 70 and 80 min, and observed the side effects such as excitement, conjunctival flushing, convulsions, poisoning, vomiting and so on, until the dog can stand or even walk. Recording baseline physiological parameters, which was comprised of Mean Arterial Pressure (MAP), Respiration Rate (RR), Diastolic Arterial Pressure (DAP), Heart Rate (HR), Systolic Arterial Pressure (SAP), Oxygen saturation (SpO_2). Experimental animals were instrumented through the non-invasive patient monitor (S/5TM; Datex-Ohmeda Drive, Madison, Wisconsin, USA) for calculate SpO_2 and RT. Only when the pulse rate obtained by the pulse oximeter matched the heart rate calculated by the stethoscope can the parameter of SpO_2 be acceptable. MAP was surveyed with "non-invasive" by the same monitor. Rectal temperature was surveyed an electronic temperature surveying instrument and it was assessed by the number displayed on the instrument. HR was determined through a stethoscope placed at the left lower lateral pleural wall by counting heartbeats for 1 min. RR was reckoned by naked eye observation or hand exploration of the number of pleural ripple cycles for 1 min.

Anaesthesia Scores Determination

Time taken for the palpebral reflex, pedal reflex and tail clamp reflex to disappeared, which was taken notes after administered anesthetic agents. The tail clamp reflexes were as similar as the reflex disappeared to nipped interphalangeal skin of a limb and the tail during 3 sec using Kocher's forceps. Resumed times to head exercise, standing and walking were written down either.

Assessment of anaesthesia refer to the proposed scoring methods *(Table 1)* [5]. Total score was the sum of posture score, sedation score, analgesia score, jaw & tongue relaxation score and auricular response score.

Statistical Analysis

Every dog should have an independent anaesthesia note. Data are exhibited in the form of mean ± standard deviation (SD) and were analyzed using: two-way analyses of variance (ANOVA) for data of several measures for HR, RR, SpO_2, MAP, SAP, DAP; the Student-Newman-Keuls or Bonferroni's test to determine overall differences at each time point in each anaesthesia group. Compared with anaesthesia groups of the score of assessment at the same time point, utilized the Fisher's Least Significant Difference test. Statistic differences were considered statistically significantly as $P<0.05$. Statistical software SPSS v 23.0 for Windows was used to summarize and analyze all test data.

RESULTS

Scores of analgesia, sedative effects, muscular flaccidity as well as posture were recordedfor each time interval. As given in the second sequence *Table 2*, in terms of total scores, conspicuous changes came up on account of two treatments, monitoring period, dog's posture, sedation, ear reaction in IA group revealed a good state of anaesthesia at 5th min of induction, and adequate muscle relaxation at 10th min of anaesthesia. But there was a lack in analgesia, determined by clamping interphalangeal skin for 3 seconds using Kocher's forceps, 75% of dogs reacted in varying degrees. In EI group, there were no apparent reactions in the dog's posture, sedative and analgesic effects 5 min after induction of anaesthesia, except a few dogs showed mild reactions (head movement) when clamping interphalangeal skin 3 sec using Kocher's forceps

Table 2. *Anaesthesia score table in total (X±SD, n=8)*

Time (min)	IA group	EI group
0	0.00±0.00	0.00±0.00
5	10.63±1.68a	13.63±1.40b
10	11.13±1.35a	15.25±1.40b
20	14.50±0.92	15.38±1.06
30	14.13±1.30	15.50±1.31
40	14.38±0.92	16.00±1.60
50	14.50±1.30	16.00±2.50
60	14.63±1.19	16.00±2.39
70	5.75±1.28	6.50±0.93
80	0.00±0.00	0.00±1.68

The contrast between the two groups, shoulder subscript letters indicate significant difference (P<0.05)

Table 1. *Criteria used to score after injected one of two groups anaesthetic agents*

Criteria	Score	Observation
Posture score	0	Normal
	1	Ataxic, but able to walk
	2	Completely prone, unable to walk,but able to crawl
	3	Lateral recumbency, but able to move the tail or paw
	4	Complete lateral recumbency without movement
Sedation score	0	Normal
	1	sternal recumbency, head movement, blinking
	2	recumbent, head down, moderate eyelid reflex, bulbus oculi enstrophe
	3	no movement, palpebral reflex disappeared, even mydriasis
Ear reaction (beat the desktop next to the ear using Kocher's forceps)	0	Normal response
	1	Unresponsive, head move with body move
	2	The body does not move but the eyes move
	3	No response reflex
Analgesic scores (Clamp Interphalangeal skin 3s using Kocher's forceps)	0	Normal response
	1	Reduced response
	2	Faint response
	3	No reflex
Muscle relaxation score (open the mouth)	0	Normal resistance to open the mouth
	1	The jaw can be opened, but there is still some resistance
	2	Little resistance to open the mouth and obvious muscle relaxation
	3	No resistance

Table 3. *Cardiopulmonary parameters of IA group and EI group (n=8, X±SD)*

Time (min)	HR (beats/min)		RR (/min)		SpO_2 (%)		SAP (mmHg)		DAP (mmHg)		MAP (mmHg)	
	IA Group	EI Group	IA Group	EI Group	IA Group (%)	EI Group (%)	IA Group	EI Group	IA Group	EI Group	IA Group	EI Group
0	120.38±9.31	118.13±11.43^{A}	22.13±0.61	23.88±0.89^{A}	98.25±1.08	97.75±0.91^{A}	127.38±3.75	128.63±4.02^{A}	75.75±3.55	73.50±4.02^{A}	96.25±4.98	94.13±6.75^{A}
5	132.50±10.24*	126.25±9.05*^{A}	20.00±0.00	25.25±1.01^{B}	98.88±0.98	96.88±0.86^{A}	131.63±4.98	128.75±3.82^{B}	78.63±2.13	74.00±2.10^{A}	98.75±6.89	94.38±3.89^{A}
10	122.25±7.87	120.00±8.32^{A}	20.00±0.00	24.13±1.78^{B}	97.75±0.98	95.75±0.79^{A}	123.25±4.29	124.13±5.09^{A}	74.38±2.09	72.88±3.64^{A}	94.38±7.53	90.25±7.26^{A}
20	118.75±6.54	118.25±7.68^{A}	20.00±0.00	20.75±0.98^{A}	98.25±0.82	95.88±0.74^{A}	120.50±3.74*	120.25±3.28^{A}	72.25±1.99	70.50±3.21^{A}	92.13±4.62	89.75±4.81^{A}
30	117.38±5.87	117.00±8.16^{A}	20.00±0.00	18.88±0.79*^{A}	99.00±0.79	95.13±0.83^{A}	116.88±5.12*	118.50±4.65*^{A}	69.88±3.72*	67.88±2.78^{A}	88.88±5.39*	88.50±5.26^{A}
40	115.50±6.42	115.88±7.36^{A}	20.00±0.00	19.00±0.63*^{A}	98.38±0.83	95.38±0.89^{A}	116.75±4.02*	115.38±3.77*^{A}	66.88±4.58*	66.38±3.46*^{A}	86.63±4.29*	86.63±6.24*^{A}
50	113.88±7.31	113.88±8.28^{A}	20.00±0.00	18.75±0.49*^{A}	99.25±0.75	95.63±0.96^{A}	116.13±4.24*	115.00±2.79*^{A}	64.25±2.76*	64.75±2.65*^{A}	86.00±8.12*	86.25±3.11*^{A}
60	113.25±5.98	111.25±9.83^{A}	20.00±0.00	18.50±0.87*^{A}	98.88±0.93	95.25±0.77^{A}	115.88±3.36*	114.63±4.58*^{A}	63.25±3.19*	64.00±3.61*^{A}	85.88±7.32*	86.00±3.23*^{A}
70	118.63±4.79	116.50±8.17^{A}	22.63±1.26	22.88±0.61^{A}	99.25±1.01	96.88±0.98^{A}	126.88±3.11	127.88±4.39^{A}	74.25±3.01	73.88±4.15^{A}	96.25±3.22	93.75±6.51^{A}
80	121.00±6.98	118.25±5.42^{A}	22.88±0.82	23.75±0.98^{A}	98.63±0.97	98.25±0.63^{A}	127.00±5.41	127.50±2.99^{A}	75.50±2.42	73.50±2.77^{A}	96.00±6.13	94.63±4.77^{A}

** datum comparison: denote the differences compares with T_0 were extremely significant, P<0.05. The different upper cases of the superscript mean that the data of the two groups were significantly different at P<0.05. A represent inconspicuous difference P>0.05, B represent significant difference P<0.05*

but the others revealed good anesthetic effects, and this state was maintained until the end of anaesthesia.

The results of cardiopulmonary parameters of IA group and EI group were summarized in *Table 3*. The HR of the two groups increased significantly (P<0.05) between 0-5 min after anesthesia, and then decreased gradually. The alteration was close to the normal values 20 min after anesthesia. From induction to the end of anaesthesia, HR of these groups had no discrepancies. In IA group, values of RR had no significant changes during anaesthesia. In EI group, the RR increased significantly during first five min after administration, then the RR began to decrease at 10th min and continued to decline until 30 min, remained stable from 30 to 60 min. Values of RR returned to normal ranges in a short time at the end of anaesthesia. Discrepancies that were overt came up (P<0.05) between two groups in RR at 5 min and 10 min. SpO_2 had been maintained at 97-100% in IA group. In EI group, SpO_2 was maintained at above 95%. During anaesthesia, mucous membranes of only a few dogs had slight color changes to white. Values of SAP, DAP and MAP were slightly higher after induction of anaesthesia, then decreased in both sets. In terms of these indicators, diversities which were obvious did not take place in both groups.

DISCUSSION

In terms of the quantity of advantages, the method of intravenous administration of volatile anaesthetics has even more advantages than the way of inhalation. Anaesthesia induction will be quick with the anaesthesia circuit rapidly and pulmonary functional residual ability is bypassed. The vaporizer which aims to set the concentration of the anaesthetic would be weed out, hence, the charge that is applied to get, as well as maintain the equipment which is used for volatile anaesthetic could be decreased. When newer inhalation anaesthetics are in use, this can be salutary in particular. Reports related to volatile anesthetics used intravenously point out that whether they occur accidentally in humans or animals, they are currently causing death or morbidity [2,3,6]. Rats as well as dogs are both the objects of halothane injection through the veins in the state of emulsion in intralipid [2,3,7] Anaesthesia for dogs via intravenous injection isoflurane were made a comparison with the way of narcotizing dogs by inhaled isoflurane. The anaesthetic using in an inhaled manner that used most far-ranging, currently in practice of veterinary medicine is isoflurane [8]. We found that 8% of EI in the aspect of induction and recovery, the first mentioned of two was faster, the latter was quieter, and in terms of chemical inhibition, it was also efficacious in dogs.

Traditionally, measurement of the depth or level of anaesthesia chiefly relies on the observation that there is an incision in the skin of humans or the tail or the interphalangeal skin of animals is clamped, a painful irritation [9]. In the present study, the assessment had to be done with the depth or level of anaesthesia depending on the method of scoring, which in group of EI was higher than that of IA groups. Scores of anaesthesia in EI group showed better analgesic aspect than IA groups when clamping interphalangeal skin for 3 secondsusing Kocher's forceps. The value of HR augmented without delay, under the circumstances of putting both of the treatments into effect and returned to near-normal at the end of the anaesthesia. This may be due to the augmentation of the velocity of heart has something to do with isoflurane [1]. Isoflurane will decrease total autonomic nervous system activity during anaesthesia, a momentous system of getting command of nervous with a view to hold the stabilization of cardiovascular is the nervous system which is autonomic [10]. No significant discrepancies were achieved under circumstance of using EI group to

compare IA group. Although the HR decreased with the continuing of anaesthesia; HR in both groups was within physiologic acceptable limits.

Similar to HR, RR increased immediately after the administration in EI group. After five min RR decreased and reached the lowest levels from 30 min to 60 min. This may be due to isoflurane has been related to increased heart rate and would be resulted in a drop off in the dose, which was followed by a decline in the rate which have to do with the respiration of dogs [11]. The decline relies on dosage would occur in the aspect of blood pressure, cardiac output as well as systemic vascular resistance of dogs, sheep, goats and humans on account of the impact to cardiovascular which generated by isoflurane in line with the report [3].

The presence of discrepancy in the matter of statistics is none when come down to SAP, DAP and MAP that pertain to both of the agents on the basis of the outcomes of this study. By reason of being short of irritation which made by operation, led to a decline of SAP, DAP as well as MAP in the state of narcotizing by both isoflurane and EI anaesthetics for a period of 10 to 60 min. In spite of transient descend in MAP within five min, these parameters became nearly normal after anaesthetic drugs. There were no significant differences among these values. Some studies verified that the stability of hemodynamic of lipid emulsion was facilitated by injecting through the way of vein of isoflurane [11,12]. IA group in contrast to EI group is a little higher in the aspect of density of SpO_2, nevertheless there were no statistical differences noted between two groups, the lower of SpO_2 concentration in EI group may be due to the way of elimination of EI. Both groups were within normal reference ranges. While injecting EI by vein, it draw a conclusion that MAC is less, in the meantime, the usage of isoflurane is even less in the case of contrasting with in drawing the steam of isoflurane [13]. In the case of the two agents, dramatic differences did not occur in the wake time of the dogs. The depth of anesthesia can be determined by 8% EI through anesthesia injection, and then the infusion speed is adjusted by the concentration of isoflurane at the end of tidal wave. Finally, the partial pressure of isoflurane in arterial blood is calculated.

As shown in *Table 3*, we found the discrepancies are not evident for HR, RR, SAP, DAP, MAP and SpO_2 between the two anaesthetics, but EI which consisted in this study, developed anaesthesia with sufficient analgesia, muscle relaxation as well as inexistence of complication to dogs. There are many links in the process of using inhalation anesthetics leaking to the external environment, causing pollution and endangering human health. Moreover, some inhalant anesthetics have irritating effects on respiratory tract at high concentrations, or they can be absorbed by ventilator. EI is a type of intravenous anesthetic which may be useful clinically for the induction of anaesthesia. Some studies demonstrated that anesthetic induction and recovery produced by EI were more rapid than that by propofol. EI has been demonstrated to protect many organs including heart, brain, spinal cord, lung, kidney and liver against the injury induced by ischemia or ischemia-reperfusion in animal models [13-16]. The EI that was used in this study provided an adequate anaesthesia effect in dogs, which was characterised by adequate analgesia and muscle relaxation without any complications. Considering that the organ protection and lower cost which belongs to intravenous EI, EI is a real innovation, it is not only a novel administrative protocol for isoflurane, but also with some unquestionable advantages. The results of our study revealed that no conspicuous differences were discovered between intravenous emulsified isoflurane and inhaled isoflurane in dogs. This study showed that there is a good prospect for using EI intravenously in dog anaesthesia.

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Conflicts of interest

The author declares no conflict of interest

Authors Contributions

Lin Li defined the research theme, gave the conception of the research. Jing Dong and Lin Li carried out experimental part of the study. Honggang Fan and Lin Li have made supervised the analysis of the results, and contributed to the writing of the manuscript. Jing Dong and Honggang Fan were involved in drafting the manuscript and revising it critically for important intellectual content and have made a substantial contribution to conception and design, analysis and interpretation of data. All authors discussed the results and contributed to the final manuscript.

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Research Article

Investigation of Correlations Between Clinical Signs and Pathological Findings in Cats and Dogs with Inflammatory Bowel Disease

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Abstract

This paper compares the correlation between the clinical signs and the histopathological observations of the entire intestine in cats and dogs with inflammatory bowel disease (IBD). To perform this study, hospital records of 53 dogs and 20 cats of different sex, ages, and breed diagnosed with IBD following the histopathological criteria of the World Small Animal Veterinary Association (WSAVA) were evaluated. The results obtained in this study did show correlations between some clinical signs and the histopathological assessment of dogs and cats with IBD. Therefore, a slight association between diarrhea and lacteal dilation in the small bowel, and diarrhea and desquamation in the large bowel of dogs with IBD was seen, but no other associations were found between the rest of the lesions and symptoms. In contrast, cats only showed a correlation between anorexia with villous stunting and villous epithelial injury, without correspondence among other clinical signs and lesions. The results of this study propose that the evaluation of IBD can be complicated, especially with the use of retrospective records of archived intestinal biopsies and subjective clinical and histopathologic decisions.

Keywords: *Dog, Cat, Inflammatory Bowel Disease, IBD, Lesion, Clinical signs*

İnflamatuar Bağırsak Hastalığı Olan Kedi ve Köpeklerde Klinik Bulgular İle Patolojik Bulgular Arasındaki İlişkilerin Araştırılması

Öz

Bu makale, inflamatuar bağırsak hastalığı (IBD) olan kedi ve köpeklerde bütün bağırsakların klinik bulguları ile histopatolojik gözlemleri arasındaki korelasyonu karşılaştırmaktadır. Bu çalışmayı gerçekleştirmek için, World Small Animal Veterinary Association (WSAVA)'in histopatolojik kriterlerini izleyerek IBD tanısı konan farklı cinsiyet, yaş ve ırkta 53 köpek ve 20 kedinin hastane kayıtları değerlendirildi. Bu çalışmada elde edilen sonuçlar, IBD'li köpek ve kedilerde bazı klinik bulgular ile histopatolojik bulgular arasında korelasyon olduğunu gösterdi. Bu nedenle, IBD'li köpeklerde ince bağırsakta ishal ve lakteal dilatasyon ile kalın bağırsakta ishal ve deskuamasyon arasında hafif bir ilişki görülmüştür, ancak lezyonların geri kalanı ve semptomlar arasında başka bir ilişki bulunmamıştır. Buna karşın, kedilerde diğer klinik bulgular ve lezyonlar arasında ilişki saptanmezken, sadece anoreksi ile villöz gelişim eksikliği ve villöz epitel hasar arasında bir korelasyon vardı. Bu çalışmanın sonuçları, özellikle intestinal biyopsilerinin arşivlenmiş retrospektif kayıtlarının subjektif klinik ve histopatolojik kararlarının kullanılması ile IBD değerlendirmesinin karmaşık olabileceğini düşündürmektedir.

Anahtar sözcükler: *Köpek, Kedi, İnflamatuar Bağırsak Hastalığı, IBD, Lezyon, Klinik bulgu*

INTRODUCTION

Inflammatory bowel disease (IBD) refers to a chronic gastrointestinal (GI) disease of unknown cause and ill-defined pathogenesis [1]. It is characterized by persistent or recurrent GI signs with inflammatory infiltration of the mucous membrane in the lamina propria area [2-6]. The etiology of this process is multifactorial and may be produced by

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inappropriate and uncontrolled inflammation of gut-associated lymphoid tissue against harmless environmental antigens [7]. Clinical signs are highly variable [1], and these go from diarrhea and vomiting to appetite disturbance such as anorexia or polyphagia. Nonetheless, the lack of clinical, diagnostic, histopathologic, and therapeutic standards resulted in great challenges that led to the design of specific indexes [8-11], which did not support a correlation between the severity of clinical signs and histopathological score since findings interpretation varied widely between pathologists [12-15]. Despite this fact, the numeric index generated by some of these methods has been used to help clinicians and researchers to correlate inflammatory lesions with clinical signs [1]. Thus, Jergens et al.[1] proposed a set of assessment criteria called the Canine Inflammatory Bowel Disease Activity Index (CIBDAI). The suitability of this index as a monitoring tool was determined by the correlation between the clinical index and the histopathological lesions. Interestingly, a study suggested that the histopathological criteria for the diagnosis of GI tract inflammation in dogs and cats could be inconsistent [10]. Due to these concerns, the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization group developed a simplified histopathologic monograph that pictorially and textually defined inflammatory and morphologic features in endoscopic biopsy specimens obtained from the stomach, duodenum, and colon [10]. However, due to the particular individuality of IBD, only a sparse number of reports defining the severity of clinical signs in dogs with IBD and its relation with histopathological lesions have been published [13,15,16]. Therefore, this study aimed to compare the correlation between histopathological changes and clinical signs in dogs and cats affected with IBD.

MATERIAL and METHODS

This retrospective study included hospital records of 53 dogs and 20 cats of different sex, ages, and breed diagnosed with IBD in the Small Animal Hospital of Las Palmas de Gran Canaria University and the Veterinary Hospital of Cordoba University. The study was performed from April 2016 to August 2018 following the approval of the Ethical Commission of Veterinary Medicine of Las Palmas de Gran Canaria University (agreement MV-2017/05). Criteria for animal selection were: clinical signs consistent with IBD such as anorexia, vomiting, diarrhea and weight loss (>3 weeks in duration), failure to respond to dietary (a commercially prepared select antigen or homemade diets) or symptomatic therapies alone, exclusion of other causes such as exocrine pancreatic insufficiency, infectious agents, endoparasites, neoplasia or food, and antibiotic responsive enteropathies; and histopathologic evidence of mucosal inflammation in biopsy specimens. The diagnostic evaluation in all animals with IBD consisted of medical records taken over 1 or more clinical examinations, hematological and serum biochemistry analyses, urinalysis, fecal test for parasites, diagnostic imaging, and histopathologic examination of GI mucosal biopsy specimens following the histopathologic scoring system of the WSAVA.

Seven dogs and four cats that were free of gastrointestinal signs over one or more clinical examinations, and showed normal hematological and serum biochemistry analyses, as well as free of parasites on fecal examinations, were used as a control group *(Table 1)*. Mucosal biopsies of the gut were also obtained from these animals as previously described in other studies [8,12].

Clinical Disease Activity Data

To evaluate the clinical disease activity, a retrospective assessment was performed following symptoms of the upper gastrointestinal part such as vomiting, diarrhea, anorexia (as appetite disorders) and weight loss, as well as symptoms of lower gastrointestinal signs such as hematochezia and mucoid feces.

Table 1. *Tissues sampled from small and large bowel of healthy dogs and cats*

Sampling Location	LD	CD	VEI	SQ	VS	MF	IEL	LPI
Duodenum (1)	+	-	+	+	-	-	+	+
Duodenum (1)	+	+	+	+	-	+	+	+
Duodenum (1)	+	-	-	-	-	+	+	+
Duodenum (1)	+	-	-	+	-	+	+	+
Colon (1)	+	+	+	+	+	+	+	+
Colon (1)	-	-	+	+	-	+	+	+
Cecum (1)	+	-	-	-	-	+	+	+
Duodenum (2)	+	+	-	-	-	-	-	-
Duodenum (2)	-	-	-	-	-	+	-	-
Duodenum (2)	+	-	-	-	-	+	-	-
Colon (2)	-	-	-	+	-	+	-	-

(1) Dog. (2) Cat. LD: Lacteal dilation; CD: Crypt dystention; VEI: Villous Epithelial Injury; SQ: desquamation; VS: Villous stunting; MF: Mucosal fibrosis; IEL: Intraepithelial lymphocytes; LPI: Lamina propria infiltrate

Histopathologic Examination

To perform the histopathological assessment, biopsy samples from the small and large bowel of diseased animals were used. These samples were fixed in 10% neutral-buffered formalin, dehydrated through graded alcohols, and embedded in paraffin wax. Sections (4 µm thick) were cut and stained with hematoxylin and eosin stain for histopathological examination. It was based on the morphological and inflammatory findings identified in the standard histopathologic system of the WSAVA Gastrointestinal Standardization group [12], as well as on those modifications done by Jergens et al.[10]. Therefore, for small and large bowel samples the main morphological features chosen were villous stunting, epithelial injury, crypt distention, lacteal dilation, desquamation, mucosal fibrosis, as well as intraepithelial lymphocyte and lamina propria infiltration. Clinical data and specific histopathological lesions were used and compared to search for significant associations.

Statistical Analysis

The lesions and clinical signs of the animals involved in this research were categorical variables summarized as frequencies and percentages. These percentages were compared using the Chi-square test. Statistical significance was set at $P<0.05$. The values in duodenum-jejunum-ileum as small intestine, and cecum-colon-rectum as large intestine were fused in order to check accurately the correlations between clinical signs and pathological findings of animals with IBD.

RESULTS

Histopathological lesions and clinical signs of 53 dogs and 20 cats of different breed and sex with IBD are shown in *Table 2* and *Table 3* (dogs), and *Table 4* and *Table 5* (cats).

The location-based on histopathologic lesions present in biopsy of dog specimens was as follows: upper intestinal part (n=27 cases) that included duodenum (n=24 cases), jejunum (n=2 cases) and ileum (n=1 case); and lower intestinal part (n=26 cases), with cecum (n=2), colon (n=17 cases), and rectum (n=7 cases). These patients showed highly variable histopathological lesions. Therefore, according to WSAVA guidelines, lacteal dilation was found in 37 out of 53 animals, crypt distention in 28 out of 53 animals, 24 animals showed villous epithelial injury, desquamation was found in 23 out of 53 animals, villous stunting just in 14 animals, and mucosal fibrosis in 35 animals in total *(Fig. 1. a-d)*.

In case of cats, the distribution on histopathologic lesions present in biopsy specimens was as follows: upper intestinal part (n=14 cases) that included duodenum (n=14 cases); and lower intestinal part (n=6 cases), where only colon lesions (n=6 cases) were detected. Among these cases lacteal dilation was identified in 12 out of 20 cats, crypt

Table 2. *Histopathologic assesment of small and large bowel of dogs with IBD*

Intestine	LD	CD	VEI	SQ	VS	MF	IEL	LPI
Small bowel (n: 27)	20	14	12	11	7	16	26	27
Duodenum (24)	17	12	11	10	6	14	23	24
Jejenum (2)	2	1	-	-		1	2	2
Ileum (1)	1	1	1	1	1	1	1	1
Large Bowel (n: 26)	17	14	12	12	7	19	26	26
Colon (17)	12	12	10	11	6	15	20	20
Rectum (7)	4	2	2	1	1	3	5	5
Cecum (2)	1	-	-	-	-	1	1	1

LD: Lacteal dilation; CD: Crypt dystention; VEI: Villous Epithelial Injury; SQ: desquamation; VS: Villous stunting; MF: Mucosal fibrosis; IEL: Intraepithelial lymphocytes; LPI: Lamina propria infiltrate

Table 3. *Relation between samples and clinical signs in small and large bowel of IBD dogs*

Intestine	Vomiting	Diarrhea	Anorexia	Weight Loss	Hematochezia	Mucoid Feces
Small bowel (n: 27)	16	21	7	7	1	-
Duodenum (24)	14	18	7	7	1	-
Jejenum (2)	2	2	-	-	-	-
Ileum (1)	-	1	-	-	-	-
Large Bowel (n: 26)	7	21	1	2	15	11
Colon (17)	5	17	1	1	11	10
Rectum (7)	1	3	-	1	4	1
Cecum (2)	1	1	-	-	-	-

Table 4. *Histopathologic assessment of small and large bowels of cats with IBD*

Intestine	LD	CD	VEI	SQ	VS	MF	IEL	LPI
Small bowel (n: 14)	9	10	6	6	2	14	14	14
Duodenum (14)	9	10	6	5	2	14	14	14
Large Bowel (n: 6)	3	4	2	4	1	3	6	6
Colon (6)	3	4	2	4	1	3	6	6

LD: Lacteal dilation; CD: Crypt dystention; VEI: Villous Epithelial Injury; SQ: desquamation; VS: Villous stunting; MF: Mucosal fibrosis; IEL: Intraepithelial lymphocytes; LPI: Lamina propria infiltrate

Table 5. *Relation between samples and clinical signs in small and large bowel of IBD cats*

Intestine	Vomiting	Diarrhea	Anorexia	Weight Loss	Hematochezia	Mucoid Feces
Small bowel (n: 14)	5	8	2	1	-	-
Duodenum (14)	5	8	2	1	-	-
Large Bowel (n: 6)	3	4	1	-	1	1
Colon (6)	3	4	1	-	1	1

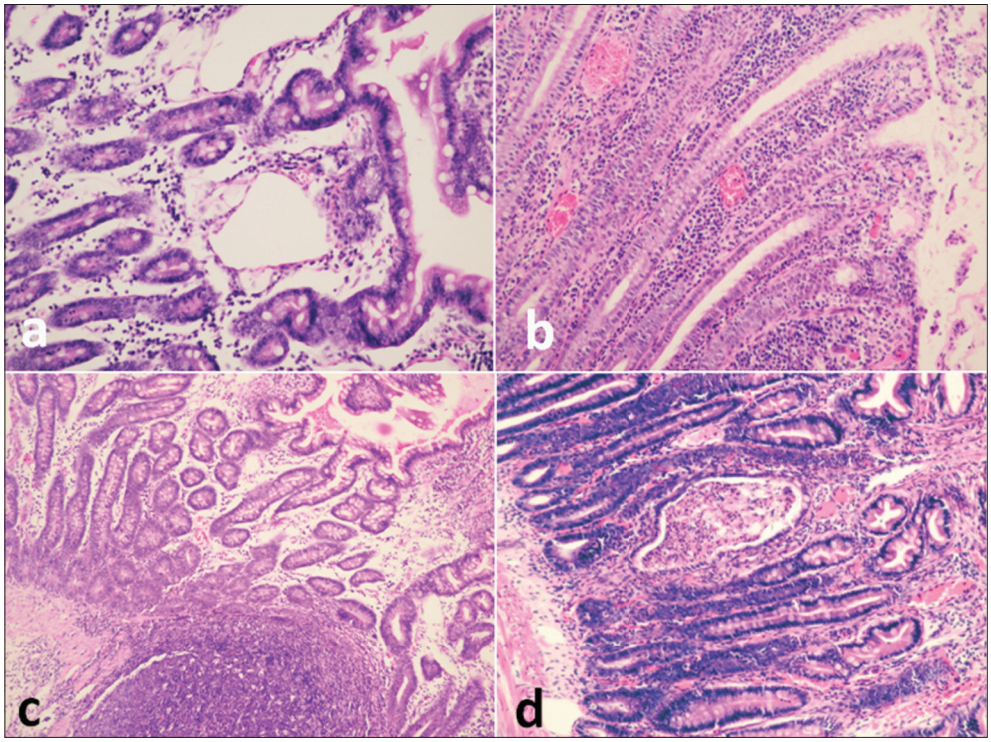

Fig 1. a) Villi appear markedly distended, with mild mononuclear infiltration, interstitial oedema of the lamina propria and moderate lacteal dilation. Haematoxylin & Eosin x200, **b)** Mild-diffuse mononuclear infiltration into the lamina propria. Haematoxylin & Eosin, x100, **c)** Mononuclear infiltration and oedema of the lamina propria, and mild hyperplasia of crypts and lymphoid aggregates. Haematoxylin & Eosin x40, **d)** Distension of a crypt, filled with mucus, inflammatory cells and cellular debris, and surrounded with attenuated epithelial cells, as well as mononuclear cell infiltration into the lamina propria. Haematoxylin & Eosin x100

distension was found in 14 out of 20 animals, 8 cats showed villous epithelial injury, desquamation was found in 10 out of 20 animals, whereas villous stunting and mucosal fibrosis were identified in 3 and 17 cats, respectively.

The cellular infiltrate in all mucosal specimens was predominantly composed of lymphocytes and plasma cells, sometimes accompanied by an admixture of sparse numbers of eosinophils, neutrophils, and macrophages. Lymphocyte infiltration in the duodenum of animals with IBD was significantly increased compared with controls, and these were mainly located in the upper part of the villi, as well as within the epithelial layer. In contrast, in the colonic mucosa, there was smaller variability between control individuals and animals with IBD.

The statistical analysis revealed a slight association between diarrhea and lacteal dilation in the small bowel (P=0.0098),

Table 6. *Frequencies and Chi-square test for the different variables considered in the small bowel and obtained from the histopathologic examination and clinical signs in dogs with IBD*

Clinical Signs	Total N =27	Lacteal Dilation		P-Value	Crypt Distension		P-Value	Villous Epithelia		P-Value	Squamation		P-Value	Villous Stunting		P-Value	Mucosal Fibrosis		P-Value
		Yes N=20	No N=7		Yes N=14	No N=13		Yes N=12	No N=15		Yes N=11	No N=16		Yes N=7	No N=20		Yes N=16	No N=11	
Vomiting	16	11	5	0.4464	9	7	0.5812	6	10	0.3811	5	11	0.2261	5	11	0.4464	11	5	0.2261
Diarrhea	21	18	3	0.0098	11	10	0.9180	10	11	0.5346	10	11	0.1736	3	18	**0.0098**	13	8	0.6007
Anorexia	7	6	1	0.4142	4	3	0.7448	1	6	0.0621	1	6	0.0979	4	3	0.0285	5	2	0.4464
Weight loss	7	5	2	0.8528	2	5	0.1521	2	5	0.3261	2	5	0.4464	2	5	0.8528	3	4	0.3048
Hematochezia	1	1	0	0.5466	1	0	0.3261	0	1	0.3621	0	1	0.3981	0	1	0.5466	1	0	0.39810

Table 7. *Frequencies and Chi-square test for the different variables considered in the large bowel and obtained from the histopathologic examination and clinical signs in dogs with IBD*

Clinical Signs	Total N =26	Lacteal Dilation		P-value*	Crypt Distensio		P-Value	Villous Epithelia		P-Value	Squamation		P-Value	Villous Stunting		P-Value	Mucosal Fibrosis		P-Value
		Yes N=17	No N=9		Yes N=14	No N =12		Yes N=12	No N=14		Yes N=12	No N=14		Yes N=7	No N=19		Yes N=19	No N=7	
Vomiting	7	5	2	0.6942	3	4	0.4951	5	2	0.1166	3	4	0.8378	1	6	0.3779	5	2	0.9084
Diarrhea	21	14	7	0.7782	13	8	0.0912	11	10	0.1918	12	9	**0.0213**	7	14	0.1310	16	5	0.4632
Anorexia	1	1	0	0.4581	1	0	0.3451	1	0	0.2707	1	0	0.2707	0	1	0.5359	1	0	0.5359
Weight loss	2	1	1	0.6341	1	1	0.9096	1	1	0.9096	1	1	0.9096	0	2	0.3716	1	1	0.4438
Hematochezia	15	10	5	0.8725	9	6	0.4623	9	6	0.0982	8	7	0.3912	4	11	0.9725	11	4	0.9725
Mucoid Fec	11	6	5	0.3198	7	4	0.3912	7	4	0.1257	6	5	0.4623	3	8	0.9725	7	4	0.3527

Table 8. *Frequencies and Chi-square test for the different variables obtained from the histopathologic examination and clinical signs in cats with IBD*

Clinical Signs	Total N = 20	Lacteal Dilation		P	Crypt Distensio, n		P	Villous Epithelia, n		P	Squamation, n		P	Villous Stunting,n		p	Mucosal Fibrosis		P
		Yes N=12	No N =8		Yes N =14	No N =6		Yes N =8	No N =12		Yes N =10	No N =10		Yes N =3	No N =17		Yes N =17	No N =3	
Vomiting	8	3	5	0.0935	4	4	0.1110	3	5	0.8522	4	4	1	2	6	0.3065	6	2	0.3065
Diarrhea	12	6	6	0.2636	8	4	0.6903	4	8	0.4561	7	5	0.3613	2	10	0.7982	9	3	0.1250
Anorexia	3	3	0	0.1250	3	0	0.2187	3	0	0.0214	3	0	0.0603	2	1	***0.0066***	3	0	0.4300
Weight lossn	1	0	1	0.2089	0	1	0.5018	0	1	0.4022	0	1	0.3049	0	1	0.6665	1	0	0.6665
Hematochezia	1	1	0	0.4022	1	0	0.5018	1	0	0.2089	1	0	0.3049	0	1	0.6665	1	0	0.6665
Mucoid Feces	1	1	0	0.4022	1	0	0.5018	1	0	0.2089	1	0	0.3049	0	1	0.6665	1	0	0.6665

and diarrhea and desquamation in the large bowel (P=0.0213) of dogs with IBD. The results of this analysis are shown in *Table 6* and *Table 7*, respectively. Concerning cats, the statistical analysis showed a high association between villous stunting (P=0.0066) and villous epithelial injury (P=0.0214) with anorexia. The results of this analysis are shown in *Table 8*, where the odds ratio with the confidence intervals is presented. No significant associations were identified among the rest of the lesions and clinical signs of dogs and cats affected with IBD.

DISCUSSION

Literature defining clinical and histopathological indexes to value the activity of the chronic inflammatory bowel disease is scarce. Therefore, its assessment in dogs and cats is quite difficult. This is particularly important in cats with a diffuse enteric disease, which exhibits mixed bowel signs and requires biopsy of both the small and large intestines for diagnosis [7]. In our study, we included hospital records that comprised biopsies of the small and large bowel. Interestingly, most of the clinical signs observed in these animals were quite similar to other studies performed on IBD that used the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) or the feline chronic enteropathy activity index (FCEAI) [1,7,15,17]. A previous study based its analysis on the intensity of different clinical signs [1]. However, in our research, the intensity and degree of the disease was partially ignored, and focused on the presence or absence of injury, avoiding interpretative ambiguity among pathologists as has been suggested by WSAVA new guidelines [10]. These subjective elements that do not always represent the existing inflammatory burden can lead to discordance between the results of different surveys using CIBDAI or FCEAI as described in other reports [13-17].

In this study, there were correlation between clinical signs and some histopathologic scores. This association was positive for lacteal dilation, desquamation, villous epithelial injury, and villus stunting. Interestingly, there was no correlation between the intensity of intraepithelial lymphocytes, lamina propria infiltrate, and mucosal fibrosis with IBD clinical signs.

Lymphangiectasia in animals is assumed to be an acquired disease, and its etiology is generally idiopathic [3]. It may also result from any type of obstruction to lymph flow in the lacteals, mesenteric lymph vessels or nodes, most frequently secondary to inflammation [8,12]. In our series, lacteal dilatation was in correlation with diarrhea, but it is important to consider that this clinical sign has been associated with chronic diarrhea [15,18]. Nonetheless, other important parameters associated with dilated lacteals such as hypoalbuminemia could not be evaluated as a consequence of the retrospective nature of our study. Other important changes observed in the mucosal architecture such as desquamation correlated well with diarrhea. A recent study indicated that changes in mucosal architecture were related to the presence and severity of GI disease [13]. Nonetheless, further prospective studies are needed to properly evaluate the meaning of these correlations.

In this study, the severity of the lymphocytic infiltration in the lamina propria did not correlate with the intensity of clinical signs. Different studies have reported that characterize the extent and severity of the inflammatory infiltrate in intestinal biopsies from dogs and cats is a difficult task [12-15]. Hence, some authors suggest that the presence of increased numbers of lymphocytes deposited in the lamina propria and their contribution to the IBD process are better explained when the intestine is checked as a big picture [8]. Moreover, studies performed in dogs and cats with IBD did show a predominant proinflammatory cytokine upregulation in the inflamed colonic and duodenal mucosa in animals with lymphoplasmacytic colitis or enteritis as happens in people, although not correlations were done [13,19]. In the present study, many similarities between the inflammatory response in the small and large intestine of dogs and cats affected with IBD were observed. Comparable findings were described in other studies [10,11,14] that showed better clinical evaluation without doing a distinction between the upper or lower intestine.

The histopathological lesions identified in the animals of this study showed significant fibrosis associated with major damage to the mucous membrane of the small and large intestine. Similar features were reported in a study done in the small intestine of animals with severe IBD [6].

Different circumstances can interfere with the histopathologic interpretation of intestinal samples such as the correct area of the GI tract to be sampled, the quality of tissue samples analyzed, and the lack of consistency in interpretation of histopathologic changes among pathologists [13-15]. These circumstances led to the WSAVA GI Standardization Group to develop a histopathologic template to avoid these concerns, but even with this histopathologic scoring system, important variations have been observed in the diagnostic interpretation of intestinal samples since the above-mentioned method did not include evaluation of all intestinal segments. Therefore, in the present study, we extended its use to other sections of the GI tract, and interestingly, its use did not show significant differences in the evaluation of the intestinal samples done among pathologists. Identical results were obtained in a recent study performed in dogs using a similar scoring system [13].

In conclusion, the histopathologic scoring system used in this study provided important information on the extent of mucosal inflammation in the GI tract of dogs and cats with IBD. However, this work proposes that the evaluation of inflammatory bowel disease can be complicated, especially with the use of retrospective records of archived intestinal

biopsies and subjective clinical and histopathologic decisions. To perform better results, it is important to follow-up on the affected animals and to evaluate outcome factors as an accurate assessment of the health status of patients as suggest reports on human chronic disease [20]. All these premises would be of great help in the uniformity of a standard for the evaluation, and monitoring of the inflammatory bowel disease.

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Statements Authors Contributions

DF, FR and JRJ designed the experiment, made the histologic interpretation, and wrote the manuscript. ARG and CF made the statistical analysis. ASB made a substantial contribution to interpretation of data. All authors discussed the results and contributed to the final manuscript.

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Research Article

The Effect of Triclosan on *In vitro* Embryonic Development in Rat [1] [2]

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Abstract

Triclosan (TCS) is a broad spectrum antimicrobial agent showing its effect by deactivating the fatty acid synthesis of bacteria. The aim of this study was to investigate the effects of TCS on *in vitro* embryonic development in rats and to determine the levels of caspases 2, 7, 8, and 9 inducing cells to apoptosis through gene expression. According to the TCS dose added to the culture whole rat serum, 3 experimental groups and a control group were formed with each including 10 embryos. After 48 h culturing period, embryos were subjected to morphological scoring for developmental evaluation. The levels of caspases 2, 7, 8, and 9 were measured by performing gene expression on 40 embryos. Significant decreases were obtained in all parameters of morphological scoring in the experimental groups as the dose of TCS increased. While the caspase-2 level showed a significant decrease among the groups and was inversely proportional to the level of TCS, the caspase-9 level showed a significant increase among the groups and was directly proportional to the level of TCS. In conclusion, TCS was determined to cause apoptosis in the cells via the intrinsic pathway during pregnancy period and lead to embryonic growth retardation, which increased with increased dose of TCS.

Keywords: *Triclosan, Rat embryo culture, Apoptosis, Gene expression, Caspase*

Triklosanın *In vitro* Embriyonik Rat Gelişimi Üzerine Etkisi

Öz

Triklosan, bakterilerin yağ asidi sentezini bozarak etkisini gösteren geniş spektrumlu bir antimikrobiyal ajandır. Bu çalışmanın amacı, triklosanın ratlarda *in vitro* embriyonik gelişim üzerindeki etkisini araştırmak ve gen ekspresyon yöntemi ile hücreleri apoptoza sürükleyen kaspaz 2, 7, 8 ve 9 değerlerini belirlemektir. Kültür şişesine eklenen triklosan dozuna göre, kontrol ve 3 deney grubu oluşturuldu. 48 saatlik kültür periyodundan sonra embriyolar gelişimsel değerlendirme amacıyla morfolojik skorlamaya tabi tutuldu. Ardından 40 embriyoya gen ekspresyonu yapılarak kaspaz-2, kaspaz-7, kaspaz-8 ve kaspaz-9 değerleri ölçüldü. Morfolojik skorlamaya ait tüm parametrelerde istatistiksel olarak anlamlı bir gerileme tespit edildi. Kaspaz-2 değerlerinin triklosan miktarı ile ters orantılı bir şekilde, gruplar arasında istatistiksel olarak anlamlı bir azalma gösterdiği, kaspaz-9 değerlerinin ise triklosan miktarıyla orantılı bir biçimde istatistiksel olarak anlamlı bir artış gösterdiği tespit edildi. Bu verilere göre, gebelik döneminde triklosan kullanımının, hücrelerde intrinsik yol aracılığıyla apoptoza yol açarak, embriyonik gelişme geriliğine neden olduğu, bu durumun doza bağlı olarak arttığı belirlendi.

Anahtar sözcükler: *Triklosan, Rat embriyo kültürü, Apoptoz, Gen ekspresyonu, Kaspaz*

INTRODUCTON

Triclosan (TCS) is a fat-soluble, broad-spectrum antimicrobial agent produced in laboratory in 1966. The area of its usage was first expanded into antibacterial soaps and then into products such as toothpastes, cosmetics, and deodorants in the following years [1]. TCS can pass the biological barriers because of its lipophilic property

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and can aggregate in living organisms [2]. The target organs have been reported to be the liver, spleen, brain, heart, reproductive system, and the immune system. Compared with other well-identified target organs, high concentrations of TCS up to 7 ng/mL can be detected in the placenta, cord blood, and amniotic fluid, which directly indicate a high risk of adverse effects on the embryo in an utero [3]. TCS is a relatively small compound with a molecular weight of 289.5 g/mole [4]. Owing to its molecular weight, TCS can pass the placenta and affect the embryo. TCS metabolism mainly occurs in the liver; however, it can also be metabolized in the skin in small amounts [5]. TCS is metabolized to glucuronide and sulfate conjugates (phase II metabolism) and primarily excreted by urine [6]. As a result of metabolism, it passes to body fluids, breast milk, and urine [7,8] and then merges into sewerage system via wastes, which causes wide contamination in the environment, such as in drinking water and surface waters [1,9]. Therefore, people using TCS-containing products are directly at risk and individuals drinking water contaminated with TCS are indirectly at risk. Many cell culture studies have shown that TCS is cytotoxic to human hepatocytes and cardiotoxic to cardiomyocytes, has neurodegenerative effects on rat neural stem cells, and shows strong embryotoxic effects on bone development [3,10-12]. However, only a few studies have reported its embryotoxic effects on embryo culture [4,13,14]. Moreover, the exact mechanism of the effects of TCS on early embryonic development is still not well understood. In a study with zebrafish embryos, growth retardation has been detected in the embryos exposed to 300 µg/L of TCS [13]. Another study has also demonstrated embryotoxic effects of TCS at high doses (250 µg/L) in zebrafish embryos, such as abnormal phenotypes, short tails, heart edema, and decreased hatching rate [4]. Developmental parameters are proved to be excellent indicators of TCS toxicity [4].

Developmental delay in babies has made the reliability of drugs and chemicals used in pregnancy period a current issue. The European Center for the Validation of Alternative Methods, established in 1991, has accepted the Rat Whole-Embryo Culture Test as one of the methods used to determine the teratogenic and toxic effects of chemical substances [15]. Therefore, this test is a preferred method in toxicology studies [16-19]. Previously, it was suggested that toxic substances triggered pregnancy loss, embryonic death or structural abnormalities by affecting the mechanisms that regulated normal embryonic development. However, today, it is known that apoptosis plays an important role in embryogenesis with cell proliferation and cell differentiation and that toxic substances disrupt the apoptosis pathway in the embryonic organs and cause malformations [20]. Apoptotic stimulus leads to intracellular activation of caspases. Pathways that activate initiator caspases vary with an apoptotic stimulus and there are two different pathways; extrinsic and intrinsic pathways. Extrinsic pathway is driven by caspase-8 and caspase-7. Intrinsic pathway, which is also called classical or mitochondrial pathway, is the major route to apoptotic death in mammalian cells. This step triggers caspase-9 and caspase-2. These caspases are not specifically present in the tissue [21,22].

In the present study, we aimed to investigate the direct toxic effects of TCS on embryonic growth and development in cultured rat embryos and to evaluate its possible genotoxic effects by determining the potential role of apoptosis on the toxic effects of TCS using the gene expression method. Real-time PCR is the most sensitive molecular method with high sensitivity (10^5-10^6), which has the risk of contamination and has a short lead time. The activations of caspase-2, caspase-7, caspase-8, and caspase-9, which may adversely affect cell survival, were measured to explore the mechanisms underlying the effects of TCS in embryo.

MATERIAL and METHODS

The study was approved by the Animal Care and Use Committee (Ethics Committee) of Erciyes University (date: 13.01.2016 and No: 16/012). All procedures throughout the study were carried out in accordance with the ethical issues. Wistar rats were obtained from the Clinical and Experimental Research Center in the Medical Faculty of Erciyes University.

Chemicals

TCS used in the study was provided from Sigma-Aldrich (CAS No 3380-34-5) (Lot# LRAA9502) and GAPDH (Lot# 0000054846), caspase-2 (Lot# 0000054850), caspase-7 (Lot# 0000054845), caspase-8 (Lot# 0000054847), and caspase-9 (Lot# 90017681) were obtained from Roche.

Embryo Culture and Morphological Scoring

Female rats approximately 4-10 months of age and weighing 150-250 g were paired with their male partners in cages at about 5.00 pm and left overnight. On the next day morning around 8 am, the female rats were examined regarding the presence of vaginal plugs as an indication of mating and thereby fertilization. The female rats with vaginal plug were considered as 0.5 day pregnant at noon. The conceptuses were dissected from the uteri, decidua and Reichert's membranes by general anesthesia on day 9.5 of gestation after the blood samples of the females were collected from the abdominal aorta. The conceptuses were explanted into whole embryo culture by the method of New [23].

In order to assess the toxic effect of TCS on embryonic growth, the embryos were divided into four groups (each consisting 10 embryos); three experimental groups and one control group. In the literature, no study of TCS dose to be applied in embryo culture was found. Therefore, the

administration dosages of TCS were determined according to the data gained from previous studies and the reference dose was accepted as 300 ng/mL [4,13,24]. The control group embryos were cultured in whole rat serum (WRS) and the experimental groups were cultured in WRS containing 100 ng/mL, 200 ng/mL, and 300 ng/mL of TCS, in accordance with the technique developed by New [23]. Using this method, the effects of TCS on *in vitro* embryonic development during the early organogenesis period (between 9.5 and 11.5 days) were evaluated. Moreover, the embryonic development was also compared between the embryos of control and experimental groups morphologically on day 11.5 (after 48 h of culturing period) via morphologic scoring system [14]. In this scoring system, taking the growth and differentiation of different embryological features into consideration, 11.5 day embryos were evaluated by 17 parameters; each parameter was divided into 6 stages; and each stage was scored with a numerical value between 0 and 5. As morphological parameters, mean yolk sac diameter; yolk sac vessel development; allantois development; embryonic flexion; heart and caudal neural tube development; hindbrain, midbrain and forebrain development; development of eye, ear, nose and pharynx; maxillary and mandibular processes; differentiation fore and hind limbs; crown-rump length; and somite number were evaluated.

Gene Expression Stages

Tissue specific RNA isolation was not possible in immature embryos due to TCS toxicity. Therefore, we used the whole embryo to perform RNA isolation of caspases that are not specific to any tissue.

RNA isolation and cDNA synthesis: Rat embryos were placed into 500 µL of TriPure Isolation Reagent (Roche Applied Science, Basel, Switzerland) for RNA isolation. Total RNA isolation was performed using the protocol for High Pure RNA Tissue Isolation Kit (Roche Applied Science, Mannheim, Germany). Qualification and quantification of RNA samples was performed using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). RNA concentrations were assessed by optical density measurement at 260 nm and 280 nm and purity was determined by the ratio of 260/280 nm. Synthesis of cDNA from the total RNA (100 ng) was performed using random hexamer as a primer via the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (PCR) analysis: From the obtained cDNA samples, mRNA expression levels of caspase-2, caspase-7, caspase-8, and caspase-9 genes, which have roles in apoptotic pathways, were investigated using quantifier real-time PCR via LightCycler® 480 II device (Roche Diagnostics, Mannheim, Germany). The primer sequences specific to the cDNAs of the investigated genes and the Universal Probe Library (UPL) probe numbers are presented in *Table 1*. Amplifications were performed in the total reaction volume of 20 µL using cDNA, mRNA-specific primers, UPL probe, LightCycler® probe master mixture (Roche Diagnostics, Mannheim, Germany), and distilled water according to the PCR cycling program: 95°C for 10 min, then 45 cycles of 95°C for 10 sec, 60°C for 30 sec, followed by 72°C for 10 sec. The level of mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was accepted as reference for normalizing the amounts of caspase-2, caspase-7, caspase-8, and caspase-9 gene expression. The procedure was repeated three times for each concentration of TCS. The expression levels of target genes were calculated using the relative quantitation method using the software program of the LightCycler® 480 II device *(Table 1)*.

Statistical Analysis

Data were analyzed using the IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). The conformity of variables to normal distribution was assessed using the Shapiro-Wilk test, Q-Q plot, and histogram graphs. In multiple independent group comparisons, one-way analysis of variance was used for normally distributed numerical variables. In post-hoc comparisons, Tukey's test was used for normally distributed variables. The Pearson's correlation analysis was performed to determine the correlation between the levels of caspases and the morphological parameters. A P value <0.05 was considered statistically significant.

RESULTS

There was severe growth retardation in the embryos of the experimental groups as compared to the control group *(Fig. 1)*. According to the morphological scoring system, the retardation of embryonic growth and development was increased as the dose of TCS increased *(Fig. 2)*. The lower morphological scores were related with the poor

Table 1. *Assay ID numbers, Universal probe number and primer sequences of genes used in expression study*

Gene Symbol	Assay ID	Forward Primer Sequence	Reverse Primer Sequence	UPL No
Casp2	506708	5¹-GGAAATGAGGGAGCTAATCCA-3¹	5¹-GGCAGCAGGTTGAGGAGTT-3¹	129
Casp7	500441	5¹-CTCTTGCGCAAAGATGCAG-3¹	5¹-AGCAGGCTGAGTTGCTGTG-3¹	110
Casp8	504044	5¹-GCCTGAGGGAAAGATGTCCT-3¹	5¹-TCACATCATAGTTCACGCCAGT-3¹	89
Casp9	502880	5¹-CGACATGATCGAGGATATTCAG-3¹	5¹-TGCCTCCCTCGAGTCTCA-3¹	27
Gapdh	503799	5¹-CATCGTGGAAGGGCTCAT-3¹	5¹-CGCCACAGCTTTCCAGAG-3¹	158

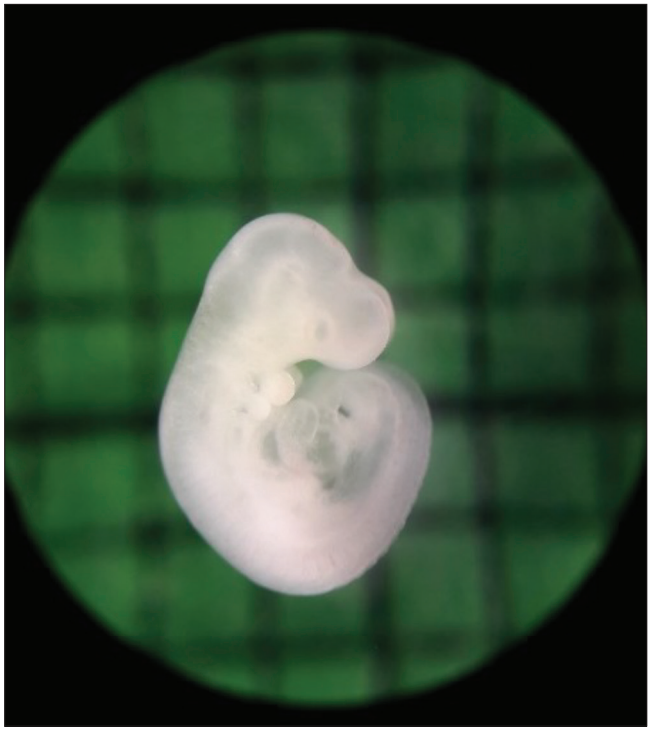

Fig 1. Normally developing embryo cultured in WRS

yolk sac vessel development, failure of fusion of the neural folds; incomplete embryonic flexion; retardation in the development of otic, optic, and olfactory systems; branchial bars; maxillary and mandibular processes; and limbs *(Table 2)*. The mean morphological scores of the embryos in the control groups was 62.80±6.42 whereas those of the embryos cultured in the WRS containing 100 ng/mL, 200 ng/mL and 300 ng/mL of TCS were 30.4±6.34, 14.9±6.55, and 1.8±3.01, respectively. The difference in the morphological scores was statistically significant between

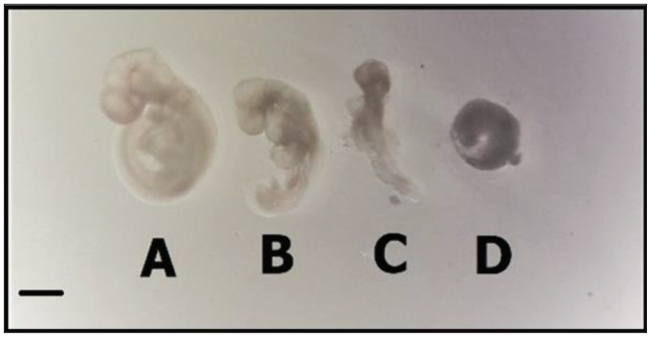

Fig 2. Embryos after 48 h culture. **A:** control group, **B:** 100 ng/mL triclosan group, **C:** 200 ng/mL triclosan group, **D:** 300 ng/mL triclosan group; Bar: 500 µm

Table 2. *Data variance analysis on morphological scoring between the control and experimental groups*

Parameters	Groups				P
	Control	100 ng/mL Triclosan	200 ng/mL Triclosan	300 ng/mL Triclosan	
Yolk Sac Vessel Development	4.8±0.422[a]	3.3±0.422[b]	1.6±0.823[c]	0.3±0.483[d]	*≤0.001*
Allantois	3[a]	1.9±0.568[b]	1±0.471[c]	0.3±0.483[d]	≤0.001
Flexion	4.9±0.738[a]	2.7±0.949[b]	1.5±0.707[c]	0.3±0.483[d]	≤0.001
Heart	4.9±0.316[a]	3±0.471[b]	1.7±0.823[c]	0.3±0.483[d]	≤0.001
Caudal Neural Tube	4.7±0.483[a]	2.4±0.516[b]	1.1±0.568[c]	0[d]	≤0.001
Hindbrain	4.4±0.516[a]	2±0.471[b]	1.2±0.632[c]	0.2±0.422[d]	≤0.001
Midbrain	4.1±0.738[a]	1.8±0.632[b]	0.9±0.316[c]	0.2±0.422[d]	*≤0.001*
Forebrain	4.1±0.738[a]	1.6±0.516[b]	0.9±0.316[c]	0.2±0.422[d]	*≤0.001*
Otic System	4.1±0.568[a]	2.2±0.789[b]	1±0.667[c]	0[d]	*≤0.001*
Optic System	4.5±0.527[a]	2.3±0.675[b]	1±0.816[c]	0[d]	*≤0.001*
Olfactory System	2.7±0.483[a]	1.1±0.316[b]	0.4±0.516[c]	0[c]	≤0.001
Pharyngeal Arch	2.9±0.568[a]	1.3±0.483[b]	0.6±0.516[c]	0[d]	≤0.001
Maxillary Processes	2.5±0.527[a]	1.1±0.316[b]	0.5±0.527[c]	0[d]	≤0.001
Mandibular Processes	2.5±0.527[a]	0.8±0.422[b,c]	0.6±0.516[b,c]	0[d]	≤0.001
Forelimbs	2±0.471	0.3±0.483	0	0	≤0.001
Hindlimbs	1.9±0.316	0.1±0.316	0	0	≤0.001
Somits	4.8±0.422[a]	2.5±0.707[b]	0.9±0.738[c]	0[d]	≤0.001
Total Morphological Score	62.8±6.426[a]	30.4±6.346[b]	14.9±6.557[c]	1.8±3.011[d]	≤0.001
Yolk Sac Diameter	3.590±0.2132[a]	2.68±0.3011[b]	1.61±0.3872[c]	1.15±0.1434[d]	≤0.001
Crown-Rump Length	2.95±0.2173	1.97±0.4057	0.86±0.2459	-	≤0.001
Somit Number	26.8±1.751	15.9±4.067	7.1±2.025	-	≤0.001

The data are as average and standard deviation. The same letters in the same row show similarity among groups, and different letters indicate differences among groups

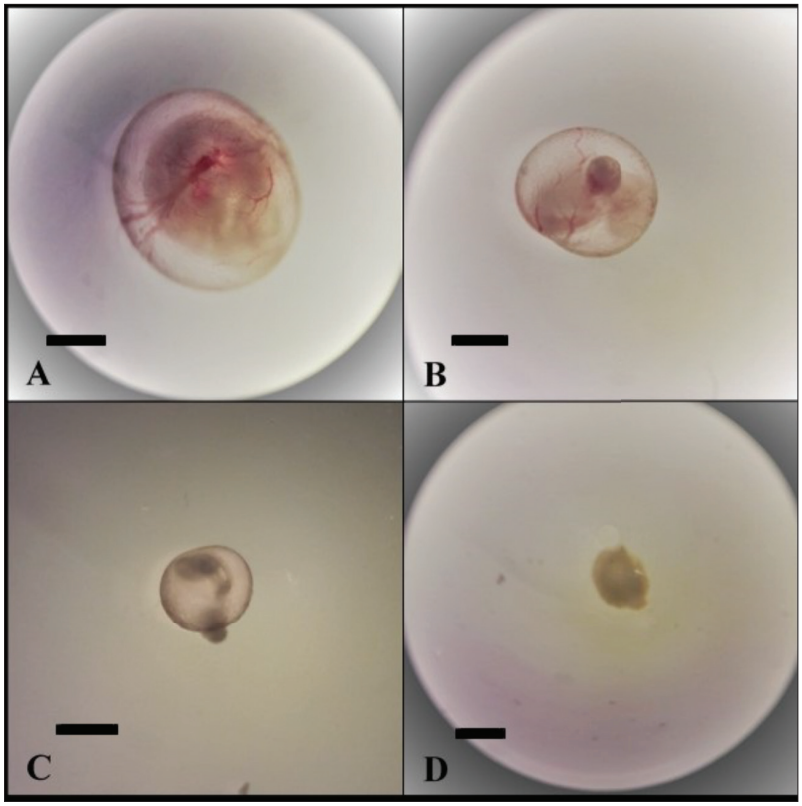

Fig 3. Yolk sac vessel development. **A:** Control group, **B:** 100 ng/mL triclosan group, **C:** 200 ng/mL triclosan group, **D:** 300 ng/mL triclosan group; Bar: 1 mm

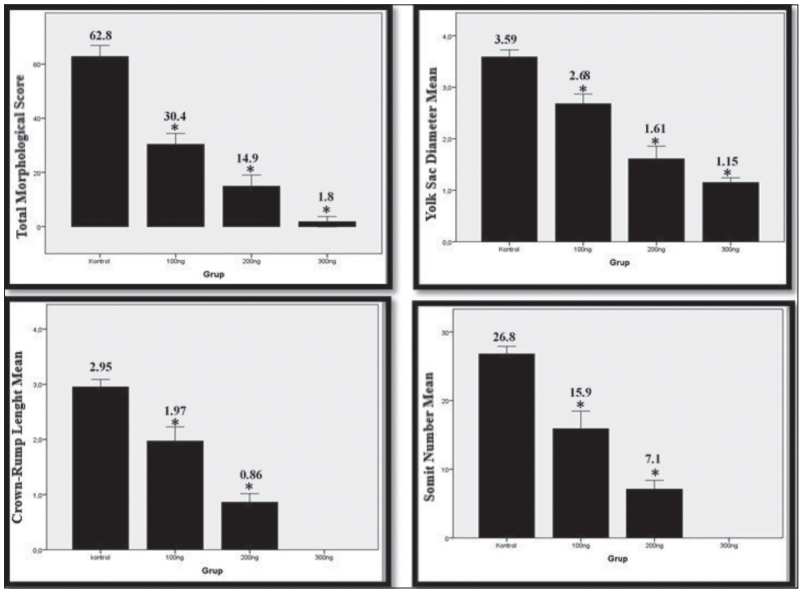

Fig 4. Effect of triclosan on total morphological score, yolk sac diameter mean, crown-rump length and somit number mean
Values are given as Mean and Standard Error, * P <0.001

the control and experimental group (P<0.001). In addition to the total morphological scores, yolk sac diameter, somite numbers, and crown-rump length were significantly lower in the experimental groups than those in the control group (P<0.001 for each). While the mean yolk sac diameter was 3.59±0.21 mm in the control group, it was 2.68±0.30 mm, 1.61±0.38 mm, and 1.15±0.14 mm in the experimental groups exposed to 100 ng/mL, 200 ng/mL and 300 ng/mL of TCS, respectively *(Fig. 3)*. The mean crown-rump length was 2.95±0.2 mm in the control group; however, in the experimental groups, it gradually diminished as the dose of TCS increased (1.97±0.40 mm, 0.86±0.24 mm, and not measured for 100 ng/mL, 200 ng/mL and 300 ng/mL, respectively). The mean somite number was 26.8±1.75 in the control group and it was also gradually diminished in the experimental groups as the dose of TCS increased (15.9±4.06, 7.1±2.02 and, not measured for 100 ng/mL, 200 ng/mL and 300 ng/mL, respectively *(Fig. 4)*. The heart development values of embryos was also lower in the experimental groups (3±0.47, 1.7±0.82, and 0.3±0.48 for 100 ng/mL, 200 ng/mL and 300 ng/mL, respectively) compared with that in the control group (4.9±0.31) (P<0.001) *(Fig. 5)*.

The molecular mechanisms underlying the toxic effects of TCS on early development of rat (whole embryo culture) was assessed via quantitative real-time-PCR and gene expression. TCS significantly altered four caspases genes (mRNA expression levels) associated with apoptosis. When caspase levels of the experimental groups were compared with the control group, TCS caused upregulation of expression of caspase-7, caspase-8, and caspase-9 and downregulation of expression of caspase-2 *(Table 3)*. There were significant differences between the experimental and control groups regarding the gene levels of caspase-2 and caspase-9 (P<0.001) *(Fig. 6)*. However, no significant difference was found between the groups in terms of the gene levels of caspase-7 (P=0.566) and caspase-8 (P=0.396). According to the correlation analysis of the levels of caspase-2, caspase-7, caspase-8, and caspase-9 with all study parameters, significant positive correlations were obtained between caspase-2 and morphological score, somite number, and yolk sac diameter. Additionally, there were significant negative correlations between caspase-9 and all morphological parameters (P<0.01).

DISCUSSION

Triclosan has been widely used in personal care products or medical devices, such as sutures, owing to its antibacterial

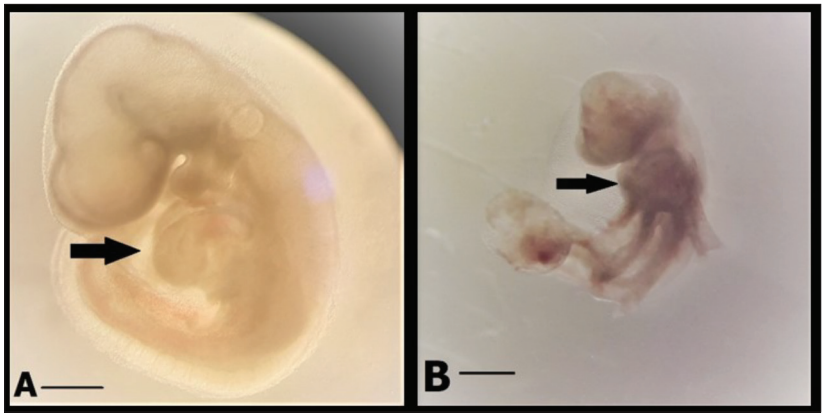

Fig 5. Heart development. **A:** control group, **B:** 100 ng/ml triclosan group, *Arrow:* Heart; Bar: 500 µm

Fig 6. Caspase levels of groups

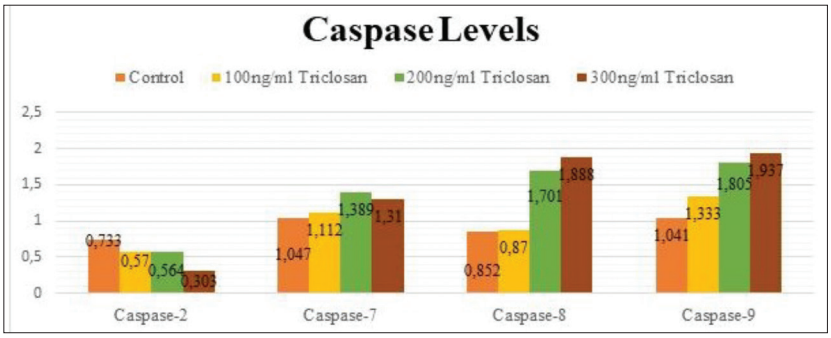

Table 3. *Mean variance analysis of experimental groups and control group*

Caspase	Groups				P
	Control	100 ng/mL Triclosan	200 ng/mL Triclosan	300 ng/mL Triclosan	
Caspase 2	0.733±0.194[a]	0.570±0.167[a]	0.564±0.166[a]	0.303±0.14[b]	<0.001
Caspase 7	1.047±0.444	1.112±0.319	1.389±0.512	1.31±0.977	>0.05
Caspase 8	0.852±0.209	0.870±0.166	1.701±0.802	1.888±3.36	>0.05
Caspase 9	1.041±0.285[a]	1.333±0.179[a,b]	1.805±0.643[b]	1.937±0.740[b]	<0.001

The data are as average and standard deviation. Different letters in the same row indicate differences between groups

properties. It is known that TCS has potential risks to reproduction and development. Although the adverse effects of TCS on different organs have been studied for decades, the mechanism of its toxicity is still not well understood, especially during the stages of embryo development [4,13]. Whole embryo culture method is a valuable model for assessing the effects of toxic agents on early embryogenesis. In this method, rat embryos are cultured *in vitro* after the gestational day 9.5-11.5, which is a critical period for organogenesis in rats and corresponds to 3-6 weeks after fertilization in human embryos. However, to the best of our knowledge, there are no studies in the literature verifying the toxic responses to TCS during embryo development in rat embryo culture. The present study highlighted the effects of TCS on *in vitro* embryonic development using cultured rat embryos and demonstrated the negative effect of TCS on cell viability leading to induction of apoptosis.

The *in vitro* embryotoxic effect of TCS using embryo/larvae of zebrafish *(Danio rerio)* was previously reported by Oliveira et al.[4]. Embryos were exposed to different doses of TCS (500 ng/mL, 700 ng/mL, and 900 ng/mL) for 6 days and embryotoxic effects (including delay in hatching, abnormal eye and body pigmentation, spinal malformations, cardiac edema, and body size smaller than normal) were observed [4]. In another study, Chen et al.[13] found no significant increase in the mortality after TCS treatment; however, 300 µg/L of TCS caused developmental retardation in zebrafish embryos at 24 h post fertilization. Ho et al.[25] examined the morphology of zebrafish larvae after TCS exposure and they observed that the zebrafish embryos exposed to 250 ng/mL of TCS developed normally, similar to the control groups. They concluded that low-dose TCS exposure did not affect general embryonic development [14]. In the study by Guo et al.[26], TCS was administrated *in ovo* to evaluate its toxic effects in the chicken embryos. Tarsus length (mm) was significantly shorter in the embryos exposed to 10 µg/g TCS compared with that in the embryos administered corn oil [26]. In another study, it was reported that exposure

to 8-10 µM TCS led to a high rate of developmental delay at 24 h post fertilization in zebrafish embryos [27]. In the present study, for the first time, effects of TCS on cultured rat embryos were examined. The results showed that the total embryonic growth was normal in the embryos cultured in the WRS only as opposed to the embryos cultured in the WRS containing TCS; this figure decreased depending on the dose of TCS. Embryonic retardations were observed in total embryonic growth, especially in the yolk sac diameter and vascularization, crown-rump length, somite number, body flexion, and neural tube development. These parameters were significantly lower in the experimental groups compared with the control group.

Researches have reported that TCS exposure can induce apoptosis in various biological systems [28-30]. In the present study, cultured embryos were also evaluated regarding apoptosis to investigate the potential role of apoptosis on the embryotoxic effects of TCS via caspases using the gene expression method. Since caspase-2, caspase-7, caspase-8, and caspase-9, which were measured to assess apoptosis, were present in all tissues, we performed RNA isolation on the whole embryo. Several studies have showed that caspase-2 is activated by DNA damage in the nucleolus [31,32]. Dubey et al.[29] reported that TCS induced apoptosis in human skin keratinocytes and responsible for DNA damage. In contrast, our data showed that TCS reduced the activity of caspase-2; if TCS had caused DNA damage, an increase in the level of caspase-2 would have occurred. Therefore, we could say that TCS did not cause DNA damage within the cell and that the decrease in caspase-2 level was associated with growth retardation. On the other hand, there is no data in the literature concerning the caspase-7 levels of TCS. Lamkanfi and Kanneganti [33] claimed that caspase-7 was related to inflammation; the activation of inflammatory caspase-1 and incorporation with caspase-7 could cause inflammation in the cell by the formation of a structure called inflammasome. Brentnall et al.[34] reported that caspase-9 and caspase-7 had different roles in intrinsic apoptosis. While intrinsic apoptosis resulted in the activation of caspase-9, caspase-7 did not have a role in intrinsic apoptosis but enabled apoptotic cell detachment [34] In the present study, as the dose of TCS increased, caspase-7 levels increased; however, no significant difference was observed between the groups. This finding suggested that TCS added to the embryo culture medium did not cause inflammation within the cell. In the *in vitro* study investigating the apoptotic effect of TCS on mouse neocortical neurons, Szychowski et al.[28] evaluated the levels of caspase-8 and caspase-9 and they found that the level of caspase-8 significantly increased as the exposure duration to TCS and the concentration of TCS increased. However, they observed the increase in caspase-9 level in the groups at high concentration of TCS. Accordingly, the researchers claimed that non-cytotoxic concentrations of TCS caused apoptosis through the extrinsic pathway; however, long-term exposure of TCS activated intrinsic pathway via caspase-9 by releasing cytochrome-c from the mitochondria [28]. In the present study, caspase-7 and caspase-8 levels increased as the dose of TCS increased; however, the difference was not statistically significant between the groups. Caspase-9 has a key role in the early stage of mitochondrial apoptosis pathway by activating downstream caspases and initiating apoptosis [35]. Li et al.[36] reported that methyl-TCS was a dominant transformation product of TCS, exposure to which induced the increased expression of caspase-9 on cell culture. In the present study, caspase-9 levels were found to be increased among the groups in proportion to TCS. It is known that TCS causes cell apoptosis via caspase-9.

In the present study, TCS significantly decreased all growth and developmental parameters in a dose-dependent manner in the embryos of the experimental groups compared with the control embryos. This finding suggests that TCS can activate the intrinsic pathway in the cell by passing directly through the cell membrane and that the intrinsic pathway is functional in the embryonic period.

In conclusion, the results of the present study revealed that TCS was a toxic agent and led to growth retardation by causing apoptosis through the intrinsic pathway without resulting in inflammation and direct DNA damage in the cell. Moreover, the current results highlighted the potential role of TCS as a strong embryotoxic in embryonic rats. These results would be an important source for the effects of TCS on embryonic development. Future studies isolating embryonic tissues and measuring the caspase levels by gene expression would also make contribution to investigate the effects of TCS on embryonic development.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

D. Çayan and E. Unur conceived the ideas of the study and writing manuscript; D. Çayan, M. Nisari, D. Patat and E. Dağlı performed data collection and analysis; H. Akalın performed gene expression stages.

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Research Article

Treatment of Traumatic Articulatio Cubiti Luxation: A Retrospective Study in Six Cats

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Abstract

Traumatic elbow joint luxation is the displacement of one or more bones forming the elbow joint due to blunt trauma, and it is uncommon in dogs and rare in cats. Although there are numerous case series in dogs, a few feline reports have been described. The aim of this study is to present the results of the clinical, radiological findings and administered treatments in cats with traumatic elbow luxation. Six cats with a diagnosis of traumatic elbow luxation due to clinical and radiological examinations were included in this study. Two acute cases were treated with closed reduction, but it was not possible to achieve reduction by close manipulation in four chronic cases. Two of the chronic cases, the lateral collateral ligament was repaired with a 2/0 polypropylene suture. The lateral collateral ligament was beyond to repair in 2 of the remaining chronic cases; in one of these cases the stability was achieved with circumferential wire prostheses, in other case with two screw and figure-eight-wire technique. There was no failure and/or complication in none of the cases after treatment. In conclusion, when the closed reduction is unsuccessful for the treatment of traumatic elbow luxation or in chronic cases, open reduction is found satisfactory.

Keywords: *Cat, Elbow joint, Luxation, Trauma*

Travmatik Articulatio Cubiti Luksasyonunun Tedavisi: Altı Kedide Retrospektif Bir Çalışma

Öz

Travmatik dirsek eklemi luksasyonu, küt travma nedeniyle dirsek eklemini oluşturan bir veya daha fazla kemiğin yer değiştirmesi olgusudur, köpeklerde karşılaşılmasına rağmen kedilerde nadir görülmektedir. Köpeklerde çok sayıda vaka serisi olmasına rağmen, kedilerde birkaç bildiri yayımlanmıştır. Bu çalışmanın amacı travmatik dirsek luksasyonu olan kedilerde klinik, radyolojik bulgular ve uygulanan tedavilerin sonuçlarını sunmaktır. Klinik ve radyolojik incelemeler sonucunda travmatik dirsek luksasyonu belirlenen altı kedi çalışmaya dahil edildi. İki akut olgu kapalı redüksiyon ile tedavi edildi, ancak dört kronik olguda kapalı manipulasyon ile redüksiyon sağlanamadı. Kronik olgulardan ikisinde lateral kollateral ligament 2/0 polipropilen sütür ile onarıldı. Diğer kronik olgularda ise lateral kollateral ligamentin onarımı yerine; bunlardan birinde çevresel serklaj ile diğerinde iki vidalı sekiz şeklinde germe teli tekniği ile stabilizasyon sağlandı. Tedavi sonrası hiçbir olguda relüksasyon veya komplikasyon görülmedi. Sonuç olarak, travmatik dirsek luksasyonunun tedavisi için kapalı redüksiyon başarısız olduğunda veya kronik vakalarda, açık redüksiyon girişimi memnuniyet verici bulunmuştur.

Anahtar sözcükler: *Dirsek eklemi, Kedi, Luksasyon, Travma*

INTRODUCTION

Elbow luxation can be congenital or occurs traumatically [1,2]. Traumatic elbow joint luxation is the displacement of one or more bones forming the elbow joint because of blunt trauma, and it is uncommon in dogs and rare in cats [3-7]. Luxation can occur because of high-energy trauma such as vehicular accidents, falls, animal fights and limb entrapments [8,9]. Because of the displacement of the bones, the normal anatomical contact of the articular surfaces of the relevant bones is disrupted [10]. Since the large medial condyle of the humerus blocks the medial luxation of the Radius-ulna, mostly the lateral luxation is seen [6,7,11]. Although there are numerous case series in dogs [8,9,12,13], a few feline reports have been described [5,6,14].

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The history usually includes trauma, mostly a vehicular accident [8]. In addition to this, it can also be caused by falling from high and cat-dog fights. The angle of the joint should be 45° or less at the time of trauma in order to allow luxation of the elbow joint without fracture [10,11].

Clinical finding is that the animal is unable to bear its weight on the affected limb and the elbow is carried in a flexed position. Palpation of the elbow reveals pain, crepitation, swelling of the joint and the resistance to flexion and extension. Two-planes radiography should be used for definite diagnosis [7,10].

Most luxated elbows can be reduced by closed manipulation if treated within the first few days after injury [3,6,15]. Closed reduction should be performed under general anesthesia [3,6,7]. The elbow joint is brought to the flexion position at an angle of 100-110° and the medial rotation of the radius-ulna is performed. After the anconeal process hooks over the lateral condyle, elbow is extended slightly. Then flexed while medial pressure on the radial head is continued. Pronation the radial head can be forced under the capitulum humeri, especially with abduction of the elbow. A straight cranio-caudal radiograph of both elbows is necessary for comparison to ascertain full reduction [7,16]. Open reduction of a luxated elbow is indicated when it is impossible to achieve closed reduction or if the joint and muscle contractures occur [7,10]. Following the general anesthesia, lateral approach to the joint is performed. A curved elevator is used for reduction. The elevator is placed under the humerus with the convex side facing upwards, inverted and pressed downwards to provide reduction of the joint. Care should be taken not to injure the joint cartilage when performing these procedures [10]. If the tension created by the triceps brachii prevents the reduction, osteotomy of olecranon can be performed [7,10,16]. Myotomy of the triceps brachii muscle can also be carried out for preventing the tension, when the olecranon osteotomy is not preferred [10]. In cases where it is not possible to repair the ruptured collateral ligaments observed during the operation, it can be replaced with two screws which are placed to lateral condyle of the humerus and caput radii and a figure-eight wire in dogs [6,7,10], or circumferential suture prostheses can be performed in cats as described by Farrell et al.[4].

After the surgery, the limb should be positioned with the elbow in extension and supported with a soft padded splinted bandage for 1-3 weeks. After removal of the bandage, passive range of motion should be performed daily, but exercise should be limited for 3 to 4 weeks [7,16].

The aim of this study is to present the results of the clinical, radiological findings and administered treatments in cats with traumatic elbow luxation.

MATERIAL and METHODS

Between June 2018 and December 2019, 6 cats were presented to the Ankara University, Faculty of Veterinary Medicine, Department of Surgery for traumatic elbow luxation. Records of all cats were reviewed for signalement, history, clinical and radiological findings and applied treatments.

Same anesthesia induction protocol was used for both open and closed reduction. Medetomidine HCl (Domitor®, Zoetis, Finland) 80 µg/kg i.m. ve Ketamine HCl (Ketasol® 10%, İnterhas, Turkey) 5 mg/kg i.m. were used for induction. Maintenance of anesthesia was achieved with isoflurane (Isoflurane-USP, Piramal, USA) &oxygen for open reduction. Perioperative cefazolin sodium (İespor®, İ.E. Ulagay, Turkey) 25 mg/kg i.v. was used for antibiotherapy. Analgesia was achieved with 0.1 mg/kg sc morphine HCl (Morphine®, Galen, Turkey).

In acute cases, reduction was achieved with closed manipulation as previously described [7,16]. In chronic cases or if the reduction was not achieved with closed manipulation, open reduction was performed via lateral approach as previously described [7]. If the lateral collateral ligament is beyond the repair, stability was enhanced by two different techniques; 1) Circumferential wire prostheses, 2) Two screw and a figure-eight wire. In the first technique, 3 bone tunnels were created with a 2 mm drill; at the humeral condyl (lateral to medial), radial head (lateral to medial) and the mid-portion of the ulnar trachlear notch (lateral to medial) as previously described by Farrell et al.[4]. Then a 0.8 mm wire was passed through the humeral tunnel, a 1.2×40 mm injector cannula was inserted to tunnel at the radial head lateral to medial, the free end of the wire was passed through the cannula, after that cannula was removed. The same procedure was applied between the humeral and ulnar notch tunnels. The four free wire ends were tightened together at the lateral side of the joint. In the second technique a 2 mm cortical screw was placed one to humeral condyl and one to radial head, then the collateral ligament was replaced with figure-eight wire which placed between these 2 screws. Except the cases which the lateral collateral ligament was replaced with one of the defined techniques above, splinted bandage was applied to the cases as the elbow joint in extension position for 3 weeks postoperatively. In the cases which an implantation is performed, soft padded bandage was applied for 3 days postoperatively. The cage rest was recommended to all cases until postoperative 3rd week. Owners were contacted through telephone for long-term follow up until 5th month postoperatively.

RESULTS

Six cats with traumatic elbow luxation were included in this study. Detailed data of the cases is presented in the *Table 1*.

Four of the cats were male and two were female. Mean age was 20.66 months (range 3 to 49 months). Four of the

Table 1. *Detailed data of the cases*

Case No	Signalement	Traumatic Cause	Clinical Complaining	Surgical Method	Outcome
1	13 month intact male DSH	TA	3 days lameness	Open reduction	S
2	11 month intact male DSH	TA	7 days lameness	Open reduction	S
3	49 month intact male DSH	TA	10 days lameness	Open reduction, circumferential suture prostheses with wire	S
4	24 month intact female DSH	FH	Unable to bear weight for 1 day	Close reduction	S
5	3 month intact male DSH	TA	Unable to bear weight for 1 day	Close reduction	S
6	24 month spayed female DSH	FH	Unable to bear weight for 8 days	Open reduction, two screw and eight-shaped wire	S

DSH: *domestic short hair,* ***FH:*** *falling from heigh,* ***S:*** *succesfull,* ***TA:*** *traffic accident*

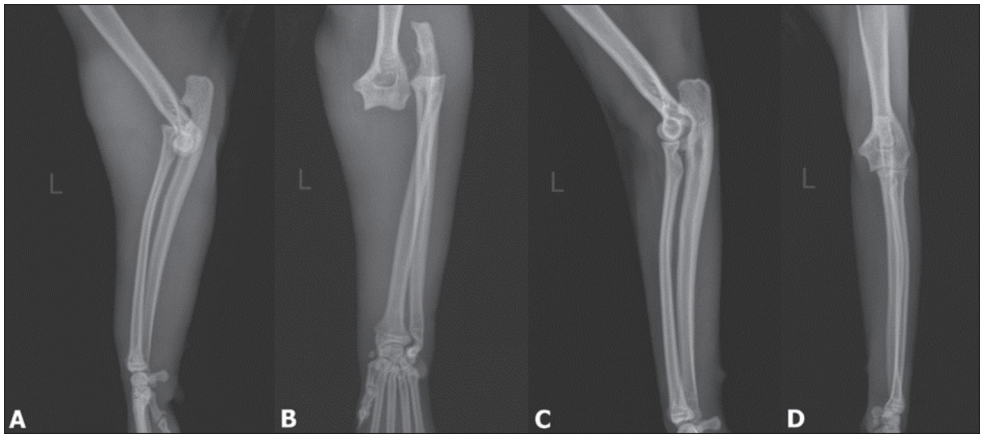
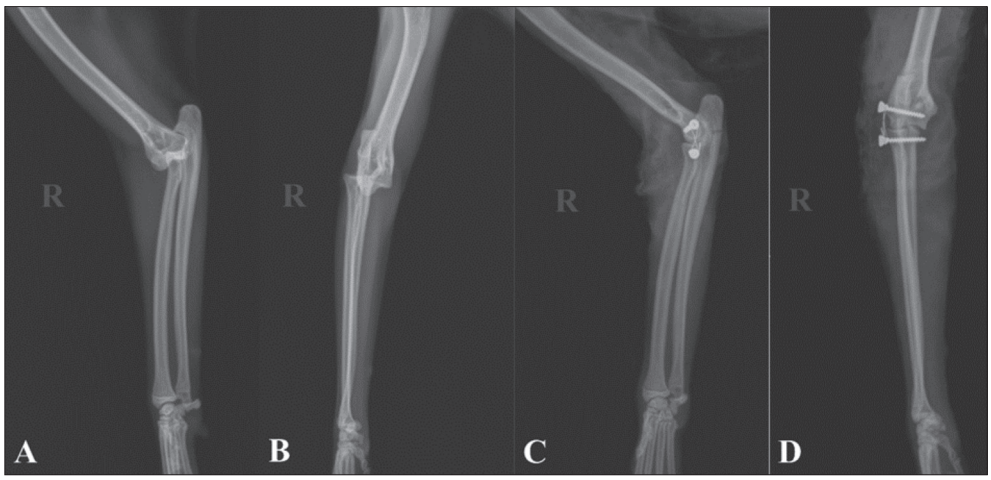


Fig 1. Preoperative (A,B) and post-operative 3rd week (C,D) radiographs of the case 1

Fig 2. Preoperative (A,B) and immediately post-operative (C,D) radiographs of the case 6



cases (case 1, 2, 3 and 5) were outdoor cats which were kept in the owners' garden.

The cause of the trauma was traffic accident in four cases and falling from height in two cases. Clinical findings were that the animal is unable to bear its weight on the affected limb and the elbow is carried in a flexed position (In case 1 and 4 left; in case 2, 3, 5 and 6 right forelimb).

Lateral elbow luxation was diagnosed by means of clinical and radiologic examinations in all cats. In all cases after general anesthesia, treatment with closed reduction was tried, but in four cases (case 1, 2, 3 and 6) it was not possible to achieve reduction with closed manipulation due to the muscle and joint contractures, so open reduction was performed. In case 1 and 2, avulsed lateral collateral ligament ends were apposed with 2/0 polypropylene suture in a locking-loop pattern, and the control x-rays that were taken immediately after the surgery showed that joint stability was successful in these two cases *(Fig. 1)*.

In case 3, the stability was achieved using circumferential suture prostheses with wire. Two screw and a figure-eight wire was used to obtain the stability in case 6 *(Fig. 2)*.

In case 4 and 5, closed reduction was performed. Because the cases were acute it was easy to achieve the reduction, control radiographs showed the stability of the joint. Although there was no complication in any of the cases after surgery, case 5 was died due to viral infection 1 month after surgery.

All cat owners except case 5 were contacted through telephone until the postoperative 5th month, no complaint related with the treated elbow joint was reported by the owners.

DISCUSSION

Dislocations of the elbow joint can occur congenitally or due to traumatic causes [6,10,16]. Although traffic accident is the most reported primary cause of the luxation in dogs, a data about the primary cause of the traumatic elbow luxation in cats is not available, because there is a small number of case reports in cats with traumatic elbow luxation [5,6]. In our study, the cause of the luxation was traffic accident in four cases (80%) and falling from height in two cases. Although there is no available case series in cats, these data is in accordance with previous literatures about the dogs. In Güzel et al.[2] study, the cause of trauma was reported as traffic accident in two of five cats and as falling from height in two of five cats. The difference between these two results may be due to the fact that four of the cats in our study were outdoor cats and they were more prone to a traffic accident.

Two-plane radiography was used for definitive diagnosis, and lateral luxation was diagnosed in all cats. The lateral luxation of the elbow is more common than medial luxation because of the fact that the medial condyle of the humerus is wide [6,7,11]. Also according to results of a cadaveric study by Farrell et al.[4], unless both medial and lateral collateral ligaments are transected, it is not possible to induce elbow luxation in cats. Also, the authors stated that the applied manual indirect rotational forces may not mimic the *in-vivo* condition as traffic accident or falling [4]. However, in our four cases which underwent the open reduction, the ruptured medial and collateral ligament were seen intraoperatively.

In this study, four of the subjected cases were chronic, so closed reduction was not successful due to joint and muscle contraction. In addition, the fibrosis of the ruptured ligaments and muscle fibrils prevented the treatment with closed reduction. In case 1 and 2 it was easy to achieve reduction despite the contraction of triceps brachii by open reduction. Nevertheless, in case 3 myotomy of this muscle was needed to perform the reduction. In case 3, a circumferential suture prostheses technique, which was defined by Farrell et al.[4], was chosen as a stabilization method. Farrell et al.[4,5] used braided polyblend suture as a prostheses, but we did not have this material in our clinics. Although we tried to stabilize the joint with No:1 polypropylene suture material, it was not possible to stabilize the joint and the suture material ruptured, and then we preferred to use wire. Although there is no comparative biomechanical or clinical study about these 2 techniques, the result of our case may show that using wire is also acceptable in some selected cases. However, it is very difficult to make a conclusion by the means of only one case's results.

A splinted bandage was applied to cases that were treated with closed reduction and cage rest was recommended for three weeks postoperatively to prevent re-luxation. Bandage application in addition to limiting the movement of the animal is suggested most of the authors, however, a simple transarticular external skeletal fixator type IIa had been applied to maintain the stability of the joint and to prevent the re-luxation in cats with caudal elbow luxation in one study [17]. Even though bandage has some disadvantages, as most of the cats do not well tolerate and mostly sedation requirement for applying, it was well tolerated by the cats in our study. Compatible temperament of the cats in this study provided an advantaged as renewing the bandage without sedation.

In conclusion, when the closed reduction is unsuccessful for the treatment of traumatic elbow luxation or in chronic cases, open reduction was found satisfactory. Even though it is not possible to claim that circumferential wire prostheses can be used in cats with traumatic elbow luxation according to results of only one case, cadaveric studies can be done to compare these two implant material (suture and wire) and to evaluate the biomechanics of wire prostheses.

Author Contributions

MS conceived and supervised the study. PC and AF collected and analyzed data, assisted MS in the operations. PC wrote the first draft of manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content and have read and approved the final version.

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Research Article

Comparison of Different Growth Curve Models in Romanov Lambs

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Abstract

This study investigates the modelling of the individual growth curves of Romanov lambs using different equations and the data on the increase in live weight and selects the best model. For this purpose, the live weights of Romanov lambs that were brought to Gürçeşme Village, Niksar, Turkey, from Nikopol, Russia, were recorded from birth to day 180. In the study, the Cubic Spline, Logistic, Gompertz and Richard models were used. For the study, individual growth curves of a total of 278 (178 females, 100 males) lambs were modeled. For the selection of the best model, adjusted determination coefficient ($R^2_{adj.}$), mean square error (MSE), Akaike information criteria (AIC) and Durbin-Watson (DW) values were used. In addition, attention was paid to the parameters and standard errors of the models. The results showed that the mean square error for the male lambs varied from 0.295 to 0.995, while it varied from 0.995 to 2.659 for the female lambs; the $R^2_{adj.}$ values were between 0.971 and 0.997 for the male lambs and 0.969 and 0.993 for the female lambs. The AIC values were between -37.12 and 0.094 for the male lambs and -0.196 and 122.12 for the female lambs. The DW values ranged from 1.86 to 2.44 for the female lambs and from 1.02 to 2.79 for the male lambs. Considering the MSE, $R^2_{adj.}$, AIC and DW values of the female lambs (0.295±1.195, 0.997±0.002, -37.12±0.001, 2.23±0.49, respectively) and male lambs (0.995±1.021, 0.993±0.001, -122.12±0.05, 2.31±0.19, respectively), the Cubic Spline model was determined to be the best model, while the Richard model was determined to be the worst fitting model both for the female (0.95±5.143, 0.971±0.002, 0.094±0.31, 2.41±0.01) and male (1.85±2.569, 0.969±0.011, -0.196±0.04, 2.79±0.05) lambs.

Keywords: *: Romanov, Lamb, Modelling, Growth curve models*

Romanov Kuzularında Farklı Bireysel Büyüme Eğrisi Modellerinin Karşılaştırılması

Öz

Bu çalışmada, Romanov kuzuların canlı ağırlık artışlarına ait veriler ile bireysel büyüme eğrilerinin farklı eşitlikler kullanarak modellenmesi ve en iyi modelin seçimi hedeflenmiştir. Bu amaçla Rusya'nın Nikopol eyaletinden Niksar Gürçeşme köyüne getirilen Romanov koyun ırkı kuzularının canlı ağırlıkları doğumdan 180. günlük yaşa kadar kayıt altına alınmıştır. Çalışmada model olarak, kubik parçalı model (cubic spline model), Lojistik model, Gompertz model ve Richard modelleri kullanılmıştır. Bu çalışma için toplam 278 (178 dişi, 100 erkek) kuzuya ait canlı ağırlık verisi kullanılmıştır. Kullanılan modeller içinde en iyi modelin seçimi için düzeltilmiş belirtme katsayısı ($R^2_{adj.}$), hata kareler ortalaması (HKO), akaike information criteria (AIC) değeri ve Durbin-Watson istatistiklerinden yararlanılmıştır. Ayrıca bu modellere ait parametreler ve standart hatalarıda dikkate alınmıştır. Elde edilen araştırma sonuçlarına göre erkeklerde hata kareler ortalaması 0.295 ile 0.995 arasında ve dişilerde 0.995 ile 2.659 aralığında, $R^2_{adj.}$ değerleri erkeklerde 0.971 ile 0.997 ve dişilerde 0.969 ile 0.993 aralığında bulunmuştur. AIC değerleri erkeklerde 0.094 ile -37.12 aralığında, dişilerde ise -0.196 ile 122.12 aralığında elde edilmiştir. DW değerleri ise dişilerde 1.86 ile 2.44 aralığında, erkeklerde ise 1.02 ile 2.79 aralığında değişim göstermiştir. Araştırma sonucunda hata kareler ortalaması, düzeltilmiş belirleme katsayısı, AIC değeri ve Durbin-Watson değerleri dikkate alındığında, dişilerde sırasıyla (0.295±1.195, 0.997±0.002, -37.12±0.001, 2.23±0.49) ve erkeklerde sırasıyla (0.995±1.021, 0.993±0.001, -122.12±0.05, 2.31±0.19) bulunmuş olup, bu sonuçlara göre kubik parçalı model en iyi model olarak tespit edilmiştir. En uyumsuz modelin ise dişilerde (0.95±5.143, 0.971±0.002, 0.094±0.31, 2.41±0.01) ve erkeklerde (1.85±2.569, 0.969±0.011, -0.196±0.04, 2.79±0.05) Richard modeli olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Romanov, Kuzu, Modelleme, Büyüme eğrisi modelleri*

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INTRODUCTION

Growth is a result of the relationship between an individual's genetic potential for the investigated property and environment. A growth curve shows the changes in yield with time due to this relationship. Yield depends on age and can be the live weight or body properties of an individual. The mathematical models showing the age-growth relationship are used to determine the nutrition program and optimum slaughter age and estimate the effects of the selection methods on farm animals. When modelling the growth, growth rate can be classified as constant growth rate, continuously increasing and decreasing growth rates and varying growth rate. Growth curves depend on the species, environmental conditions and investigated property and thus, selection of the appropriate model requires the use of statistical decision-making. Although growth at a constant rate can occur in certain properties of organisms during certain periods, it has been reported that, in general, the growth rates of organisms are not constant [1-4].

In most cases, linear models fall short in modelling the growth of organisms during an entire life period [2-5]. During periods of varying growth rates, the use of more complex non-linear models is more useful compared to linear models, in fact, it is necessary. Another important advantage of non-linear models is that they can serve as the basis of an objective method that will estimate the growth potential and sustainable production of an organism [6].

The purpose of growth curves is to summarize the information that is hard to interpret and obtained from different points due to age with lower number of parameters that can be interpreted biologically. The structure of the data and purpose of the analysis are the two important criteria used in the selection of the method that will be used in the growth curves. Moreover, the parameters of the model that will be used in the estimation of the growth curves are expected to be biologically interpretable. The biological interpretation of the parameters depends on interpreting the relationship between genetics and environment well [3].

Using the developed asymptotic and monomolecular functions, the age-growth relationship for the investigated property in lambs is estimated. In addition, the parameter values that can be the selection criteria for these models are determined [7]. In animal breeding, growth curves give important information on optimum slaughter age and economic growth threshold. Moreover, a growth model validity of which is controlled and accepted (for live weight and body measurements) can be used for the estimation of the growth at a certain period and, thus, for early selection. Growth curves allow estimating the growth of an individual in the future and, thus, selecting the animals that have good growth for breeding at an early age [2,8].

In sheep breeding in Turkey, the majority of the economic revenue is generated by lamb production. One of the major reasons behind the insufficiency to meet the need for animal products is that sheep breeding is mostly dependent on low-yield local breeds [9]. Romanov lamb is a sheep breed that was obtained in North Russia after regular improvement studies for many years and has a high ability to transfer its properties to its offspring. Compared to other sheep breeds, Romanov lambs have higher breeding capability and viability. Due to their high adaptation and breeding, they are preferred in herd breeding and, thus, data on Romanov lambs were used in the study considering their adaptability to the breeding conditions in Turkey [10].

There are various husbandry studies on the issue investigating different species [11,12]. However, the number of studies on the growth models for Romanov lambs is limited.

The study investigates the fitness of individual growth curves that were modeled using the Richard, Logistic, Gompertz and Cubic Spline models to the data for both female and male Romanov lambs. The MSE, $R^2_{adj.}$, AIC and DW values were compared in both female and male Romanov lambs for the four different growth curve models. In addition, estimations for the parameters of the individual growth curves for four different models are given.

MATERIAL and METHODS

In the study, the increases in the live weights of a total of 278 Romanov lambs (178 female and 100 male) that were brought to Niksar Gürçeşme from Nikopol, Russia, were recorded from birth to day 180 and individual growth curves were modeled.

The lambs were fed with the same ration program from weaning (2.5 months) to the end of the experiment (day 180) and the lambs were not allowed to pasture. Before weaning, each lamb was monitored individually and according to their live weights and suckling, they were daily fed with the 2500 kcal/ME and 12-16% HP-containing initial concentrate feed in an average amount of 200-300 g. After weaning, the lambs were separated from their mothers and divided into special groups according to their birthdays and, again, using the ration programs, they were fed with the 2500 kcal/ME and 18-21% HP-containing concentrate feed and high-quality roughage by paying attention to their live weights and ages and the amount of the feed was homogenously and carefully calculated to apply the necessary feeding program.

The common feature of the models used in the fitness of growth curves is their use of two main biological parameters: the performance and growth rate of an individual at a certain point, usually at a mature age. However, in some models, another parameter is the increasing or decreasing

changing point of a growth curve in terms of the growth rate. The non-linear models are commonly used in the investigation of the relationship between growth and age.

Four different functions were used for the individual growth curves in Romanov lambs, comprising the Richard, Logistic, Gompertz and Cubic Spline functions. For the sake of clarity, the α, β, k and m parameter representations of the models in *Table 1* were replaced by β_0, β_1, β_2 and β_3 respectively. For all models included in this study, the parameters are defined as β_0, the maximum potential of the asymptote or dependent variable; β_1, biological constant; β_2, controls the rate at which the dependent variable approaches the potential maximum; β_3 is allometric constant. Knots are determined by considering concave and convex formation points. In Cubic Spline, the number of the knots position is very important [13]. *Table 1* shows the mathematical models of the functions [14-17].

The NLIN (non-linear regression) procedure of the SAS 9.0 System for Windows was used in the adaptation of the growth curve models (W and β) to the live weight data and estimation of the parameters [18]. The Marquardt method was used for iteration, which was preferred due to being a representation of a reconciliation between the Gauss-Newton and Steepest descent methods and combining the best aspects of the two methods by eliminating their severe limitations [19]. The Marquardt iteration model requires parameters to be estimated and their initial values, a model with a single dependent variable and partial derivatives of the model for each parameter. The statistical methods that are known and suitable for the linear models are usually not suitable for non-linear models and F-statistics cannot be used to reach a conclusion at any significance level [19]. Thus, the models are compared to each other using unexplained variances [20].

Goodness-of-fit Criteria

Goodness of the fit of the models was evaluated using the adjusted determination coefficient $R^2_{adj.}$, mean square error (MSE), Akaike information criteria (AIC), correlation between observed live weight and residuals (RESC) and Durbin-Watson autocorrelation test (DW) [21].

The goodness-of-fit criteria to compare the functions that will explain the growth of lambs are as follows:

Table 1. *Model expressions and parameters of the growth functions*

Models	Expressions
Richard	$W_t=\beta_0/(1+\beta_1 \exp(-\beta_2 t))^{1/\beta 3}$
Logistic	$W_t=\beta_0/(1+\beta_1 \exp(-\beta_2 t))$
Gompertz	$W_t=\beta_0 \exp(-\beta_1 \exp(-\beta_2 t))$
Cubic Spline	$W_t=\beta_0+\beta_1 t+\beta_2 t^2+\beta_3 t^3+\beta_4 (t-a)^3$

W_t: *t. live weight on the day,* ***β_0, β_1, β_2, β_3, β_4:*** *Model constants describing the growth curves of the Richard, Logistic, Gompertz and Cubic Spline models,* ***exp;*** *the natural logarithm base,* ***t:*** *Age(days),* ***a:*** *knot*

Adjusted Determination Coefficient (R^2_{adj}),

The adjusted R^2 ($R^2_{adj.}$) value, which was developed by Henry Theil to avoid the increase in the R^2 value that is obtained using the explanatory variables, was used.

$$R^2 adj. = R^2 - \left(\frac{k-1}{(n-k)(1-R^2)}\right) \quad (1)$$

where *n* is the number of observations and *k* is the number of parameters.

Different than the R^2 value, adjusted R^2 ($R^2_{adj.}$) only increases when the absolute t value of the added variable is higher than 1. $R^2_{adj.}$ is always lower than or equal to R^2 ($R^2_{adj.} \leq R^2$) [22].

Mean Square Error (MSE),

$$MSE = SSE/(n-k) \quad (2)$$

where n is the number of observations, SSE is the sum square of errors and k is the number of parameters.

Akaike Information Criteria (AIC),

Akaike information criteria (AIC) are used to select the most compatible model among different models [23]. The model with the lowest AIC value is selected as the most compatible model.

$$AIC = n \times \ln\left(\frac{SSE}{n}\right) + 2k \quad (3)$$

where n is the number of observations, SSE is the sum square of errors and k is the number of parameters [24].

Durbin-Watsons (DW)

There are various tests that were developed to determine autocorrelation. When the number of observations is 13, the most commonly used test is the Durbin-Watson (DW) *d* statistics.

$$d = \sum_{t=2}^{n}(e_t - e_{t-1})^2 / \sum_{t=1}^{n} e_t^2 \quad (4)$$

The value of the *d* statistics and its comparison to the lower ($d_{LOWER}=d_L$) and upper ($d_{UPPER}=d_U$) limits in the Durbin-Watson (DW) *d* statistics table ($d_{LOWER}=d_L$) allows deciding whether autocorrelation exists or not. The Durbin-Watson (DW) value is compared with the lower and upper critical values, d_L and d_U. If the calculated DW is lower than d_L, there is a positive autocorrelation between the error terms (DW close to 0) [22]. If the calculated DW is higher than d_U, there is not an autocorrelation (DW close to 2) or there is not a negative autocorrelation between the error terms (DW close to 4). If the calculated DW is between d_L and d_U, the test is inconclusive, i.e. autocorrelation cannot be decided [25].

RESULTS

Table 2 shows the values that were calculated using the live weights and live weight gain values of both female

and male Romanov lambs and the individual growth curves obtained with the Richard, Logistic, Gompertz and Cubic Spline models. The MSE, $R^2_{adj.}$, AIC and DW values of the models were given both for female and male lambs. According to *Table 2*, the model with the lowest MSE value was the Cubic Spline model for both female and male lambs, while the Richard model and Logistic model had the highest MSE values for the female and male lambs, respectively.

Table 2 shows that the MSE values for the female lambs were 0.95, 0.534, 0.405 and 0.295, in the Richard, Logistic, Gompertz and Cubic Spline models, respectively, while the MSE values for the male lambs were 1.850, 2.659,1.369 and 0.995, respectively. Moreover, the $R^2_{adj.}$ values of the female lambs were between 0.971 and 0.997, while they were between 0.969 and 0.993 for the male lambs.

Furthermore, *Table 2* shows the AIC and DW values in different models for both male and female lambs. As seen in the *Table 2*, the lowest AIC value of the female lambs was -37.12 and obtained with the Cubic Spline method, while it was -122.12 in the male lambs and, again, obtained with the Cubic Spline model. The DW value was in the desired ranges for both male and female lambs and the values indicated no autocorrelation.

Table 3 shows the growth parameters of the female and male lambs estimated by Richard, Logistic, Gompertz, and Cubic Spline models. The highest mean β_0 parameter values (205.7 for female lambs and 214.1 for male lambs) were estimated by the Richard model. The β_0 parameter calculated with the Richard model was higher than those estimated with other models.

The β_1 parameter was estimated by all models used in the study and represents the ratio of live weight gain after birth to adult live weight. In male lambs, the highest β_1 parameter was obtained when the Gompertz model was used (5.012), followed by the Logistic, Cubic spline and Richard models. In female lambs, the highest value for the β_1 parameter was obtained with the Logistic model (2.223), followed by the Gompertz, Cubic Spline and Richard models.

Furthermore, the β_2 parameter that was commonly estimated by the Richard, Logistic, Gompertz and Cubic Spline growth curve models shows at what rate the live weight at age t approaches the adult live weight. In male lambs, the highest β_2 value giving information about the growth rate was obtained with the Gompertz (0.895) model, followed by the Richard (0.711), Logistic (0.0389) and Cubic Spline (-0.027) models. In female lambs, the highest maturation rate (β_2 parameter) was obtained with the Richard (0.842) model, followed by the Logistic (0.0228), Gompertz (0.018) and Cubic Spline (-0.006) models.

Moreover, *Fig. 1* and *Fig. 2* show the growth curves of different growth models for both female and male lambs. When the weight measurements of an organism that are taken during its life cycle or a certain period are adjusted

Table 2. *MSE, $R^2_{adj.}$, AIC and DW values and their standart error of the models*

Gender	Model	MSE	$R^2_{adj.}$	AIC	DW
Female	Richard	0.950±5.143	0.971±0.002	0.094±0.31	2,41±0.01
	Logistic	0.534±2.215	0.972±0.004	-5.41±0.05	2.44±0.69
	Gompertz	0.405±7.152	0.990±0.015	-23.44±0.07	1.86±0.33
	Cubic Spline	0.295±1.915	0.997±0.002	-37.12±0.01	2.23±0.49
Male	Richard	1.850±2.569	0.969±0.011	-0.196±0,04	2.79±0.05
	Logistic	2.659±0.476	0.990±0.009	-12.32±0.08	1.02±0.57
	Gompertz	1.369±8.978	0.986±0.002	-21.44±0.09	1.58±0.59
	Cubic Spline	0.995±1.021	0.993±0.001	-122.12±0.05	2.31±0.19

MSE: *Mean Square Error, R^2_{adj}:* ***AIC:*** *Akaike Information Criteria,* ***DW:*** *Durbin-Watson Statistic*

Table 3. *Estimations for the parameters of the growt h functions*

Gender	Model	β_0	β_1	β_2	β_3	β_4	Knot
Female	Richard	205.70±0.02	0.005±0.05	0.842±1.02	-	-	-
	Logistic	33.46±2.11	2.223±0.15	0.0228±0.01	-	-	-
	Gompertz	47.79±3.09	1.008±0.25	0.018±0.22	-	-	-
	Cubic Spline	3.35±0.21	0.106±0.01	-0.006±0.05	0.0009±0.03	-0.002±0.01	75
Male	Richard	214.10±0.01	0.045±0.15	0.711±1.89	-	-	-
	Logistic	38.45±3.19	4.213±0.05	0.0389±0.89	-	-	-
	Gompertz	41.72±1.09	5.012±0.02	0.895±0.06	-	-	-
	Cubic Spline	3.11±0.01	0.356±0.18	-0.027±0.01	0.0011±0.06	-0.027±0.04	75

to the growth models, the resultant curves usually have an S-shape and, thus, are called sigmoidal curves. Sigmoidal curves are the best models in explaining biological growth. As seen in *Fig. 1* and *Fig. 2*, although the distributions of the models and calculated values were close to each other until a certain period (until about the age of three months), they partially diverged from each other during the period until the age of six months.

This is also revealed by the $R^2_{adj.}$ values of the models. The $R^2_{adj.}$ values in the Richard model for both male and female lambs were especially lower than other models. As seen in the *Fig. 1* and *Fig. 2*, the curve of the Richard model diverged from the curves of other models.

As revealed by the curves, the curve that was closest to the real value was the curve of the Cubic Spline model, indicating that the best model was the Cubic Spline model.

DISCUSSION

The study was carried out to determine the best model among four different models by using the data on the increase in the live weights of Romanov lambs until the age of 180 days. For this purpose, MSE, DW, AIC and $R^2_{adj.}$ values were used.

In the study, four different values were primarily used to determine the best model. When the model fitness is sorted in accordance with the AIC values, the model with the lowest AIC value is accepted as "the best" model. According to the AIC, the models with an AIC value lower than 2 can be considered to have a good support [23,26].

According to the results, in females, the lowest mean square error was 0.295±1.915, the highest $R^2_{adj.}$ value was 0.997±0.002, the lowest AIC value was -37.12±0.001 and DW value was 2.23±0.49 and obtained with the Cubic Spline model, while, in males, the lowest MSE was 0.995±1.021, the highest $R^2_{adj.}$ value was 0.993±0.001, the lowest AIC value was -122.12±0.05 and DW value was 2.31±0.91 and, again, obtained with the Cubic Spline model. The results agree with the results obtained in other studies [27-31].

Sengul and Kiraz [32] reported that high R^2 values for Logistic and Gompertz models in a their study of growth curves of turkeys. Tekel et al.[33] concluded that Gompertz, Logistic

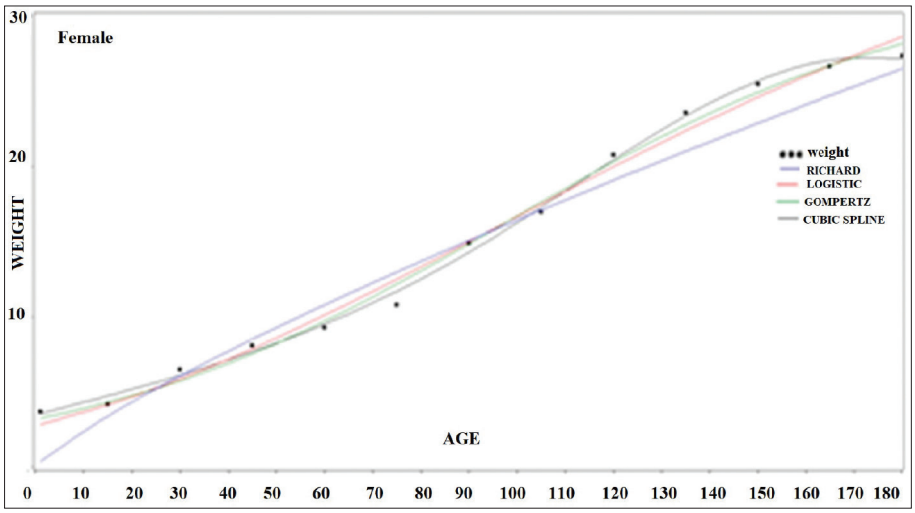
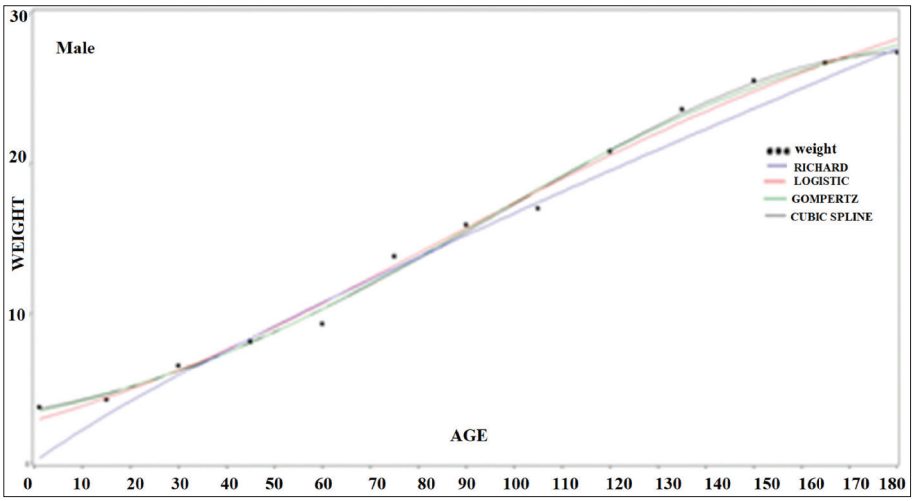


Fig 1. Growth curves of the female lambs by different growth functions

Fig 2. Growth curves of the male lambs by different growth functions



and Bertalanffy models described growth of Awassi lambs better than Brody and Negative exponential models.

Yıldız et al.[31] used the Gompertz and Logistic models on the determination of the growth curves of Merinos x Kıvırcık hybrid lambs. The determination coefficient (R^2) in the Gompertz model (R^2) was 0.986 for female lambs and 0.990 for male lambs, while it was 0.982 in the Logistic model both for the male and female lambs. In a similar fashion, in this study, the adjusted determination coefficient ($R^2_{adj.}$) in the Gompertz model was 0.990 for female lambs and 0.986 for male lambs, while the $R^2_{adj.}$ value in the logistic model was 0.972 and 0.990 for the female and male lambs, respectively.

In Aggreys study on the determination of the growth curves of poultry with different models [26]. Aggrey used non-linear models (Gompertz, Logistic, Richard) and the Cubic Spline model. The researcher found that the determination coefficient (R^2) in the Richard and Gompertz models was 0.981 for female lambs and 0.982 for male lamb, while it was 0.978 for female lambs and 0.980 for male lambs in the Logistic model; the R^2 value in the Cubic Spline model was 0.960 and 0.964 for female and male lambs, respectively. Aggrey [26] found that the Cubic Spline model had the lowest R^2 value, whereas the R^2 value in the Cubic Spline model was 0.997 for the females and 0.993 for the males in this study. This indicates that the growth curve models yield different results for different species, even for different breeds. On the contrary to the study carried out by Aggrey [26], in this study, the best model for the estimation of the growth curves of Romanov lambs was determined to be the Cubic Spline model.

Aytekin and Zulkadir [30] used the Gompertz, Logistic and Cubic Spline models to determine growth curves of Malya sheep and found that the determination coefficients were 0.915, 0.912 and 0.921 in the Gompertz, Logistic and Cubic Spline models, respectively. Their results agree with the results obtained in this study. Celikeloğlu et al.[34] used the Gompertz and Logistic models to determine growth curves Pırlak sheep and found that the determination coefficients were 0.950 and 0.942, respectively. These results agree with the results obtained in this study. Keskin and Dag [35] used the Linear and Quadratic models to determine growth curves of Anatolian Merino lambs and found that the determination coefficients were 0.990 and 0.984, respectively. The fattening periods of this study and our study are similar and there is a difference between the models used.

Balan et al.[36] used the Gompertz, Logistic and Richards models to determine growth curves of Mecheri sheep and found close R^2 values to those obtained in our study.

The study revealed that the best growth model for both male and female Romanov lambs was the Cubic Spline growth function with its lowest MSE, highest $R^2_{adj..}$ and lowest AIC values. In addition, the Cubic Spline model attract the attention as the model with the highest auxiliary values. DW value indicated that the Cubic Spline model did not have an autocorrelation problem.

The shape of growth curves varies depending on the species, environmental conditions and investigated property. In this case, there are certain factors the researcher should consider when attempting to obtain a model. The first factor is deriving a growth/time equation that will be used as the growth function from a differential equation and second factor is selecting biologically interpretable parameters for this equation [4,37].

In conclusion, considering the comparison criteria for the live weight values of Romanov lambs, the best model was determined to be the Cubic Spline model and the Richard model was determined to be the least compatible model.

We would also like to point out that the growth differences due to genotype can result in the need to use different models for the fitness of growth data. Model fitness for growth curves can vary depending both on genotype and investigated property. In the fitness of growth curves, when selecting a model, emphasis should be put on the structure of the data, ease of estimation and biological interpretability of the parameters that will be estimated.

Another factor affecting model selection is the fluctuations in live weight due to age. This can stem from the physiological differences between the individuals as well as the differences between the environments. For example, factors that cause sudden changes in live weight such as reaching sexual maturity at different periods, climate, production level, diseases and stress can affect model selection and the shape of growth curves.

Furthermore, Romanov sheep's high reproductive performance, meat yield and adaptability to the geographical conditions of Turkey render it an important breed for the breeders in Turkey. In the study, the live weight values of the Romanov lambs during a six-month period were determined to be close to those of local breeds. Cross-breeding studies can increase the reproductive performance of the local breeds without decreasing their meat yield.

Author Contributions

Y. Tahtali conceived the ideas of the study and writing manuscript; M. Sahin performed data collection and analysis; L. Bayyurt performed data analysis and writing manuscript

Conflict of Interest

The authors declare no conflict of interest

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Morphological and Genetic Characteristics of Zerdava, A Native Turkish Dog Breed [1][2]

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Abstract

Zerdava dogs are considered as one of the many native animal genetic resources of Turkey. However, the genetic characteristics of these dogs and detailed phenotypic studies related to them have not been reported yet. The aim of this study was to determine the morphological and genetic characteristics of Zerdava dogs. Blood samples (n = 100) were collected from Zerdava dogs. The morphological characteristics of these dogs were also taken. The mean live weights of Zerdava dogs were found to be 16.02±0.35 kg. The mean withers height, rump height and body length were measured as 48.20±0.21, 47.08±0.24 and 51.24±0.23 cm, respectively. According to the results of microsatellite markers, the mean F_{IS} (inbreeding coefficient) value was documented as 0.0361±0.0003. Observed (Ho) and expected heterozygosity (He) values were found to be 0.708±0.091 and 0.694±0.077, respectively. In addition, the frequency of A018 (72%) and B001 (16%) haplotypes were high in Zerdava dogs. The mitochondrial DNA sequence results show that the majority of Zerdava dogs originate from two different maternal lines. According to the results, the phenotypic and genotypic variations of Zerdava dogs were low. Therefore, these results may suggest that Zerdava dogs may have been protected by local breeders and can be considered a separate breed.

Keywords: *Genetic characteristics, Dog, Morphological characteristics, Turkey, Zerdava*

Türkiye'nin Yerli Bir Köpek Irkı Olan Zerdava'nın Morfolojik ve Genetik Özellikleri

Öz

Zerdava köpekleri Türkiye'de birçok yerli hayvan gen kaynaklarından biridir. Ancak, bu köpeklerin genetik ve ayrıntılı fenotipik özellikleri hakkında çalışmaya rastlanılmamaktadır. Bu araştırmanın amacı, Zerdava köpeklerinin morfolojik ve genetik özelliklerini belirlemektir. Zerdava köpeklerinden kan örnekleri (n = 100) alınmıştır. Köpeklere ait morfolojik özellikler de incelenmiştir. Zerdava köpeklerinin ortalama canlı ağırlığı 16.02±0.35 kg olarak bulunmuştur. Ortalama cidago, sağrı yüksekliği ve vücut uzunluğu sırasıyla 48.20±0.21, 47.08±0.24 ve 51.24±0.23 cm'dir. Mikrosatellit işaretleyicilerinin sonuçlarına göre, ortalama F_{IS} (nispi katsayı) değeri 0.0361±0.0003. Gözlenen (Ho) ve beklenen heterozigot (He) değerleri sırasıyla 0.708±0.091 ve 0.694±0.077 olarak tespit edilmiştir. Buna ilave, Zerdava köpeklerinde A018 (%72) ve B001 (%16) haplotiplerinin görülme sıklığı yüksek bulunmuştur. Mitokondriyal DNA sekans sonuçları çoğu Zerdava köpeğinin iki farklı anne soyundan köken aldığını göstermektedir. Mevcut sonuçlara göre, Zerdava köpeklerinin fenotipik ve genotipik varyasyonları düşük olduğu belirlenmiş olup bu köpeklerin yerel yetiştiriciler tarafından korunmuş olabileceği düşünülmektedir.

Anahtar sözcükler: *Genetik özellikler, Köpek, Morfolojik özellikler, Türkiye, Zerdava*

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INTRODUCTION

Zerdava dogs have been grown and raised in the Eastern Black Sea Region, especially in Trabzon and Giresun provinces of Turkey for more than at least a hundred years. This genotype is well known as the gate dog or Eastern Black Sea Spitz. Nowadays, these dogs are owned for hunting (e.g. fox, jackal, badger and pigs), guarding, search and rescue aims. In terms of temperament, they are known to have brave, fearless, aggressive and stubborn characteristics [1]. Hence, Zerdava dogs can be shown as a candidate dog to meet the military and police dog needs of our country.

Morphological characteristics are important in distinguishing and explaining the genetic relationship among breeds [2-7]. The body of Zerdava dogs is of medium size and has a colour, varying from light chestnut to dark chestnut. However, white hairs can be seen in some parts of the body. The whiteness at tail is one of the most important morphological features of these dogs. In general, they have small round spots on the white on their legs. The colours of the spots are the same with the body colours. These dogs, which have adapted to living in mountainous regions, are small and have an athletic structure. The physical characteristics of these dogs include upright ears, deep and wide chest and inward-curved tail, which is covered with longer hairs relative to those in the body [1,8].

Because of their features, Zerdava dogs have been grown locally in the Black Sea region of Turkey for many years [1,8]. In recent years, an increasing interest has been developed for these dogs in many regions in Turkey. This situation may cause uncontrolled genotype crossings and disruption of genotype characteristics. Thus, these dogs should be identified morphologically and genetically and should be protected. However, the genetic characteristics of these dogs and detailed or enough phenotypic studies related to them have not been reported. The aim of this study is to determine the morphological and genetic characteristics of Zerdava dogs.

MATERIAL and METHODS

Samples

In this study, 100 (50 females and 50 males) blood samples were collected from twelve month and older (Mean mature live weight: fourteen months) Zerdava dogs from Trabzon (Latitude-longitude: 41′ 0145-39′ 7178) and Giresun (Latitude-longitude: 40′ 2811-38′ 8953) provinces in the Eastern Sea region of Turkey *(Fig. 1)*. Trabzon and Giresun are neighbouring provinces and have a rainy climate that is unique to the Black Sea region. The study was conducted by the consent of Ethics Committee of Ankara University (protocol no: 2013-18-135). All animals that had been included in this study had an owner. The animals were hosted individually in kennels. In general, mature dogs were fed with home-made meals containing meat once a day, although the diets differ according to their owners. These animals were the offspring of the parents who had the all characters of the breed. During mating, the females in estrus were kept in the male's kennel for three days. All dogs were selected based on morphological standards and on information about their pedigree. The data about the animals were collected from Zerdava dog clubs and associations (Trabzon Zerdava Dog Association). The samples were collected from non-relative individuals that are thought to be best representatives of their breed characteristics. The blood samples collected from the animals were placed into 10 cc tubes containing anti-coagulants (EDTA) from *Vena cephalica antebrachii* of the dogs. The samples were stored at -20°C until DNA isolation.

Morphological Studies

For morphological studies, the live weight and head and body measurements were taken as indicated in the literature [2]. The significances of differences among gender groups were identified by a t-test, whereas those among age groups were discerned by a one-way analysis of variance. Duncan's test was performed for multiple comparisons of age groups, SPSS software was used for the statistical analysis of data [9].

Genetic Studies

DNA Isolation: A DNA isolation kit (Thermo Scientific Co., California, USA) was used for the DNA isolation of the blood samples. The procedure was carried out in accordance with the manufacturer's recommendations. The absorbance values of the DNAs were measured with a spectrophotometer at 260 and 280 nm. All samples were stored at -30°C until polymerase chain reaction (PCR) analysis.

Amplification and Analysis of Microsatellite Markers: Thermo Scientific Canine Genotypes Panel 1.1 kit (Thermo Scientific Co., California, USA) was used for the amplification of microsatellite markers from the DNA samples. PCR amplification and imaging were performed in accordance with a previous study [10].

The mean heterozygosity values of the dog population were carried out according to the method of Nei [11] whereas the F_{IS} values and deviations in the Hardy-Weinberg equilibrium were estimated using the Genetix 4.05 program [12].

A factorial similarity analysis (FSA) was performed to show the relationship between breeds, where the Genetix 4.05 computer package program was used [13].

Mitochondrial DNA d-loop Analysis: The amplification of the mitochondrial DNA (mtDNA) control region (d-loop) was performed by a PCR analysis [14]. The DNA sequences were edited using Sequencher 5.4.5 (Gene Codes Corporation, Ann Arbor, MI, USA), such that the sequences

were arranged with a length of 582 base pairs (bps). The sequence was then aligned with ClustalX 1.81 and BioEdit 7.0.9 sequence alignment program. The sequences of five samples were submitted to GenBank (Zerdava-TRZ.1-3 and Zerdava-GRS.1-2). Nucleotide differences (π) among the dog populations, population mutation rate (Θ) and Tajima's D values were identified using MEGA 4 computer package program [15].

RESULTS

The live weight, body measurements and statistical values of Zerdava dogs in the gender and age groups are presented in *Table 1*. The mean live weight values of Zerdava dogs were 16.02±0.35 kg. The mean withers height, rump height and body length were found to be 48.20±0.21, 47.08±0.24 and 51.24±0.23 cm, respectively. The mean head length was recorded as 20.95±0.09 cm, the face length was 8.49±0.05 and the ear length was 10.04±0.07 cm. The withers height, rump height, chest depth, chest circumference, live weight, shank circumference, distance between the ears, ear length and mouth circumference were statistically significant depending on age. Moreover, the withers height, rump height, body length, chest depth, chest circumference, shank circumference, head length, face length, ear length, tail length and mouth

Fig 1. Zerdava dogs and provinces where the samples are collected from

Table 1. *The statistic values ($X\pm S_x$) for live weight (kg) and some morphological measurements (cm) of Zerdava dogs*

Groups	n	Withers Height	Rump Height	Body Length	Chest Width	Chest Depth	Chest Circumference	Front Cannon Circumference	Back Cannon Circumference	Live Weight
Age		*	**	-	-	***	**	*	**	*
12 month	27	47.03±0.40^{a}	45.64±0.45^{a}	50.52±0.44	16.69±0.33	19.04±0.22^{a}	50.92±0.68^{a}	8.73±0.10^{a}	8.26±0.11^{a}	14.31±0.58^{a}
13-24 month	34	48.45±0.36^{b}	47.52±0.45^{b}	51.85±0.39	17.73±0.29	20.60±0.20^{b}	53.53±0.61^{b}	9.13±0.09^{b}	8.63±0.10^{ab}	15.72±0.53^{ab}
25-36 month	20	48.44±0.48^{b}	47.46±0.53^{b}	51.08±0.52	17.82±0.38	21.16±0.26^{b}	54.44±0.81^{b}	9.14±0.12^{b}	8.84±0.13^{b}	16.74±0.85^{b}
37+ month	19	48.88±0.48^{b}	47.71±0.54^{b}	51.50±0.53	17.77±0.39	21.22±0.02^{b}	54.30±0.82^{b}	9.01±0.12^{ab}	8.54±0.13^{ab}	17.88±0.96^{b}
Gender		***	***	***	-	***	**	***	**	-
Female	50	47.23±0.31	46.12±0.35	50.42±0.34	17.34±0.25	20.13±0.17	52.48±0.54	8.82±0.08	8.43±0.08	15.79±0.51
Male	50	49.17±0.29	48.05±0.33	52.06±0.32	17.66±0.24	20.88±0.16	54.11±0.50	9.18±0.07	8.70±0.08	16.25±0.47
Total	100	48.20±0.21	47.08±0.24	51.24±0.23	17.50±0.17	20.50±0.12	53.30±0.37	9.00±0.05	8.57±0.06	16.02±0.35
Groups	**n**	**Head Length**	**Face Length**	**Ear Length**	**Ear Width**	**Distance Between Ears**	**Distance Between Eyes**	**Mouth Circumference**	**Tail Length**	**Whiteness Length at Point of Tail**
Age		-	-	*	-	*	-	***	-	-
12 month	27	20.57±0.18	8.40±0.09	9.78±0.13^{a}	8.50±0.11	10.49±0.23^{a}	4.06±0.27	17.34±0.19^{a}	28.79±0.58	6.49±0.54
13-24 month	34	21.00±0.16	8.57±0.08	10.36±0.12^{b}	8.84±0.10	10.81±0.20^{ab}	4.86±0.24	18.81±0.17^{c}	28.81±0.51	7.00±0.48
25-36 month	20	21.15±0.21	8.56±0.11	10.00±0.16^{ab}	8.85±0.14	11.37±0.27^{b}	4.32±0.31	18.16±0.22^{bc}	28.85±0.68	6.99±0.64
37+ month	19	21.07±0.22	8.43±0.11	10.01±0.16^{ab}	8.74±0.14	11.48±0.27^{b}	4.69±0.32	18.04±0.23^{b}	28.73±0.69	7.17±0.65
Gender		***	***	**	-	-	-	*	***	-
Female	50	20.56±0.14	8.31±0.07	9.85±0.10	8.67±0.09	11.18±0.18	4.26±0.21	17.78±0.15	27.90±0.45	6.78±0.42
Male	50	21.34±0.13	8.67±0.07	10.22±0.10	8.80±0.08	10.89±0.17	4.70±0.19	18.40±0.14	29.69±0.42	7.04±0.40
Total	100	20.95±0.09	8.49±0.05	10.04±0.07	8.73±0.06	11.04±0.12	4.48±0.14	18.09±0.10	28.79±0.31	6.91±0.29

*- P>0.05; * P<0.05; ** P<0.01; *** P<0.001 ^{a,b,c}Means within columns with different superscripts differ significantly (P<0.05)*

Table 2. *Heterozygote (He and Ho) indexes. Hardy-Weinberg equilibrium and F_{IS} values in Zerdava dog populations*

Lokus	Heterozygote Indexes			Hardy-Weinberg Equilibrium		F_{IS}
	Allele Numbers	He	Ho	P*	Significance	
AHT121	12	0.790	0.781	0.044	*	-0.003
AHT137	10	0.765	0.766	0.000	***	-0.005
AHTh13	10	0.769	0.800	0.000	***	-0.053
AHTh171	10	0.682	0.620	0.000	***	0.133
AHTh260	9	0.765	0.800	0.000	***	0.002
AHTk211	6	0.724	0.713	0.616	-	0.045
AHTk253	5	0.646	0.657	0.243	-	-0.024
CXX279	9	0.679	0.635	0.084	-	0.052
FH2001	9	0.811	0.730	0.333	-	0.149
FH2054	9	0.745	0.701	0.004	**	0.061
FH2328	9	0.812	0.774	0.015	*	0.069
FH2848	10	0.749	0.810	0.000	***	-0.083
INRA21	7	0.718	0.650	0.006	**	0.113
INU005	8	0.650	0.669	0.218	-	-0.037
INU030	7	0.787	0.803	0.087	-	-0.002
INU055	9	0.687	0.672	0.000	***	0.021
LEI004	5	0.735	0.752	0.007	**	-0.071
REN105L0	6	0.802	0.723	0.002	**	0.135
REN162C04	6	0.685	0.699	0.097	-	-0.033
REN169D01	10	0.806	0.672	0.000	***	0.207
REN169O1	8	0.741	0.745	0.764	-	0.007
REN247M2	6	0.498	0.467	0.000	***	0.033
REN54P11	8	0.779	0.737	0.000	***	0.032
REN64E19	6	0.750	0.721	0.304	-	0.081
$h_S \pm S_e$		**0.732±0.071**	**0.712±0.077**			**0.0361 ± 0.0003**

*- P>0.05; * P<0.05; ** P<0.01; *** P<0.001; Mean heterozygosity and Standard error ($h_S \pm S_e$)*

circumference differences were statistically significant depending on gender.

For the genetic analysis, the DNA samples of 100 dogs were analysed using 24 polymorphic microsatellite loci. In the microsatellite loci used in the study, the most alleles were AHT121 loci (12 alleles) and the least alleles were LEI004 and AHTk253 loci (five alleles) *(Table 2)*. Some microsatellite loci are not in the Hardy-Weinberg equilibrium *(Table 2)*. The heterozygosity indices (He and Ho) and mean heterozygosity values of the studied dog populations are presented in *Table 2*. The mean He and Ho values were 0.732±0.071 and 0.712±0.077, respectively. According to the t-test results, the difference between the calculated mean heterozygosity values was not statistically significant (P>0.05).

For the determination of population differentiations, an F_{IS} test was performed for each locus, and the results are shown in *Table 2*. The F_{IS} value of the analysed populations was 3.61%, which is the coefficient of inbreeding and exhibits the average heterozygote deficiency for each population.

In the study, a 582 bp mtDNA d-loop control region was used to determine the present haplotypes in the study group. According to mt-DNA D-loop region, the distributions (%) of A011, A018, A020, A028, A178, B001, B042 and C003 haplotypes were 4%, 72%, 2%, 1%, 1%, 16%, 2% and 2%, respectively. In the dog populations, five haplotypes were identified in haplogroup A, and two haplotypes were found in haplogroup B. The frequency of A018 (72%) and B001 (16%) haplotypes was high.

The results of the FSA are given in *Fig. 2*. The results detected the presence of two distinct groups in the studied group. There was no relationship between the groups and the provinces where the samples were collected.

The total number of polymorphic regions, polymorphic region ratio, nucleotide differences, population mutation ratio and Tajima's D values were calculated for the nucleotide sequences of the dog populations examined. In the study, eight different polymorphic regions in the mtDNA control region of the populations were determined, and the polymorphism rate was 3.5%. The mutation rate,

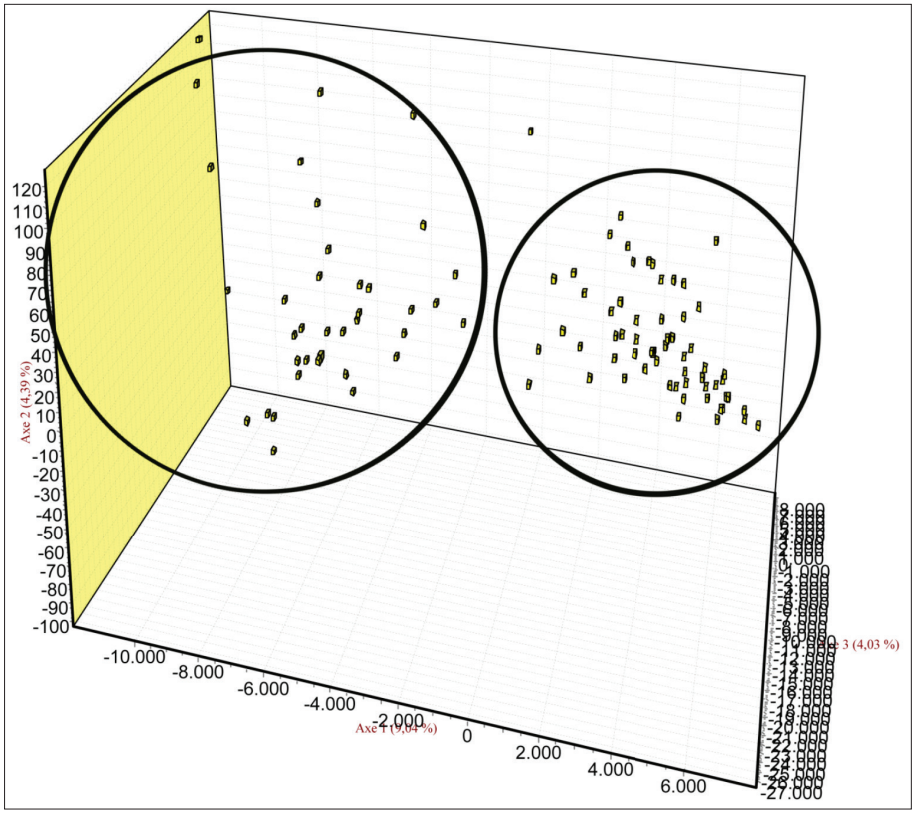

Fig 2. Factorial similarity analysis in the study group (The each circle represents more closely related animals according to genetic similarity)

nucleotide difference and Tajima's D value were 0.006, 0.009 and 1.416, respectively.

DISCUSSION

A previous study was conducted to determine the morphological characteristics of Zerdava dogs [1]. In the study on Zerdava dogs, the withers height, rump height, body length, chest circumference, chest width, shank circumference and head length were 51.2, 51.6, 56.3, 50.1, 25.6, 9.4 and 19.4 cm, respectively. In the present study, the live weight values were 16.02±0.35 kg. The mean withers height, rump height, body length and head length were 48.20±0.21, 47.08±0.24, 51.24±0.23 and 20.95±0.09 cm, respectively. The values obtained from this study for Zerdava dogs are lower than those obtained by Yilmaz and Ertugrul [1]. These differences may be because, the previous study was carried out with a small number (Total 39 dogs) of dogs and conducted with samples in a single province (Trabzon) and a limited group.

The genetic characteristics of some Turkish dog breeds have been reported in previous studies [10,16,17]. However, no studies have been conducted for the genetic characterisation of Zerdava dogs. Therefore, this is the first study performed on the genetic characterisation of Zerdava dogs.

Microsatellite markers are one of the standard tools used for the molecular genetic characterisation of breeds [17,18]. Altunok et al.[17] used microsatellite markers to determine the genetic differences among Kangal dogs and other Turkish dog breeds. They reported that the He values were 0.743 in the Kangal breed, 0.620 in the Akbaş breed and 0.705 in the Turkish hounds. Erdogan and Ozbeyaz [16] analysed the polymorphic loci from 276 dogs to determine the genetic relationship among some dog breeds. The mean heterozygosity was estimated between 0.32 and 0.41, and the difference in heterozygosity among dog breeds was insignificant (P<0.05). Although, the heterozygosity determined in the present study (0.732±0.071) was similar to that reported in the study carried out by Altunok et al.[17], but the values are higher than those reported in other studies [16,18,19].

The deviations in the Hardy-Weinberg equilibrium and the genetic differences among the subpopulations provide information about the genetic structure of the population [13]. The F_{IS} (inbreeding coefficient) parameters in Wright's F-statistics model are also commonly referred to as associated with *homozygous index*. In the present study, the microsatellite marker analysis results show that the average F_{IS} was 0.0361±0.0003, and the observed (Ho) and expected heterozygosity (He) indices were 0.732±0.071 and 0.712±0.077, respectively. The t-test results show that the difference between the average heterozygosity calculated in the present study was statistically insignificant. The deviations in the Hardy-Weinberg equilibrium and F_{IS}, Ho and He values suggest that populations can be heterogeneous in terms of the loci examined, closed breeding is less applied and non-relative individuals in the sample were selected successfully. A similar situation is seen more clearly when the FSA *(Fig. 2)* is taken into

consideration. According to this graph, the Zerdava dogs are classified into two groups.

mtDNA has been used as a genetic marker in population genetics for many years [14,18,20,21]. The sequence analysis of the mtDNA d-loop region can detect point mutations in the early stage of replication. Thus, information about maternal evolution can be obtained through an mtDNA sequence analysis in a population. Dog mtDNA haplogroups are divided into six main phylogenetic groups (A, B, C, D, E and F), and the percentage of dogs in these groups are 71.2%, 18.0%, 7.6%, 2.5%, 0.3% and 0.3%, respectively [18,22]. Most of the dogs are in one of the three major haplogroups. Almost all of the old-world dog populations have similar haplogroup rates (A, 55%-85%; B, 10%-35%; and C, 5%-15% [23]. In addition to haplogroups A, B and C, haplogroup D is common in 2.3% of dogs in Southwest Asia. Haplogroups E and F were detected in East Asia [24]. The haplogroups C and D have been detected from Central and West Europe, including France, Germany, Switzerland, and Hungary. On the other hand, haplogroups D, A and C are common in Eastern Europe, Iran and the Middle East [25-27]. The three haplogroups (A, B, and C) can be determined in Kenyan dogs, while Nigerian dogs are in one of four haplogroups (A, B, C, and D) [28]. In this study, the mtDNA sequence analysis found that Zerdava dogs were identified in haplogroups A, B and C. The frequency of A018 (72%) and B001 (16%) haplotypes was found to be high.

The Tajima's test is one of the most commonly used tests to determine mutations and natural selection of populations [29]. In the present study, eight different polymorphic regions were detected in the mtDNA control region, and the polymorphism rate (ps) was determined as 3.5%. The mutation rate *(Θ)* in the population, nucleotide difference and Tajima's D values were 0.006, 0.009 and 1.416, respectively. These results indicate that the population may have a low number of mutations, continuity of breed purity, balanced selection of samples and sampling from limited populations.

This research was conducted to determine the morphological and genetic characteristics of Zerdava dog, which is one of Turkey's native genetic resources. According to the data obtained, the phenotypic and genotypic variations of Zerdava dogs are very low, and thus it can be said that the genotype has been preserved by local breeders. In addition, the findings of this study show that Zerdava dogs may have sub-varieties. For the next stage, necessary steps should be taken for the protection of this breed.

Statement of Author Contributions

FA and FTÖ designed the study and manuscript preparation. The sampling, data collection and laboratory analysis were made by ME, BYÖ, BÖ and FTÖ. All the authors read the manuscript.

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Research Article

The Impact of an Essential Oil Mixture on Growth Performance and Intestinal Histology in Native Turkish Geese *(Anser anser)*

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Abstract

This study investigated the impact of an essential oil (EO) mixture (Biomin) on growth performance, carcass yield, visceral organ weights, and duodenal crypt depth in native Turkish geese *(Anser anser)*. Sixty 1-day-old goslings constituted the animal material of the trial. Goslings were divided into three treatment groups, each consisting of four subgroups having five animals each. Animals were homogenously distributed among the groups after being weighed and identified. The three treatment groups received the essential oil mixture at rates of 0.0% (without EO), 0.1% and 0.2%, respectively. The animals were given concentrate feed (21.93% CP and 3010 kcal/kg ME) for the first 2 weeks, and were both grazed and provided with barley meal for the next 8 weeks. The trial was continued for a period of 10 weeks. Results showed that the essential oil mixture had no significant effect on live weight gain, feed intake, feed conversion rate, carcass yield, and heart-liver-gizzard weights (P>0.05). On the other hand, the crypt depths of the EO 0.1% and EO 0.2% groups were greater than that of the EO 0.0% group (P<0.05). This study demonstrated that EO supplementation had no impact on performance, but increased duodenal crypt depth in native Turkish geese.

Keywords: *Crypt depth, Essential oil, Native Turkish goose, Performance*

Yerli Türk Kazlarında *(Anser anser)* Esansiyel Yağ Karışımının Büyüme Performansı ve Bağırsak Histolojisi Üzerine Etkisi

Öz

Bu çalışmada esansiyel yağ (EY) karışımının (Biomin) yerli Türk kazlarında besi performansı, karkas randımanı, bazı organ ağırlıkları ve duodenum kript derinliği üzerine etkisi incelenmiştir. Hayvan materyali olarak 60 adet 1 günlük Yerli Türk kazı *(Anser anser)* kullanılmıştır. Kazlar üç deneme grubuna ve her birinde beş hayvan bulunan dört alt gruba ayrılmıştır. Hayvanlar tartılarak ve ayaklarından numaralandırılarak homojen dağıtılmıştır. Esansiyel yağ, gruplara sırasıyla; %0.0 (EY'siz), %0.1 ve %0.2 düzeylerinde verilmiştir. Hayvanlar iki hafta konsantre yemle (%21.93 HP ve 3010 kcal/kg ME) sekiz hafta mera+arpa kırmasıyla beslenmiştir. Deneme 10 hafta sürmüştür. Araştırmada esansiyel yağ karışımının canlı ağırlık artışı, yem tüketimi, yem dönüşüm oranı, karkas randımanı, kalp, karaciğer, taşlık ağırlıkları üzerine anlamlı bir etkisi olmamıştır (P>0.05). Diğer yandan EY %0.1 ve %0.2 gruplarındaki hayvanlara ait kript derinlikleri, EY %0.0 grubuna göre daha yüksek çıkmıştır (P<0.05). Yapılan bu araştırma yerli Türk kazlarında EY karışımının hayvanların performansına herhangi bir etki yapmazken, duodenum kript derinliğini artırdığını göstermiştir.

Anahtar sözcükler: *Esansiyel yağ, Kript derinliği, Performans, Yerli Türk kazı*

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INTRODUCTION

The intensive and indiscriminate use of antibiotics in the poultry sector has led to antibiotic residues in food products of animal origin, and the emergence of cross-resistant bacteria. These risks, which are both directly and indirectly linked to human health, have impelled researchers to investigate alternative growth factors [1]. Since the ban of the use of antibiotic growth promoters (AGPs) in food animals in the European Union (EU) complying with EU law, aromatic herbs and herbal products (oil, powder) have attracted the attention of the poultry sector as feed additives alternative to antibiotics [2-5]. Essential oils (EOs) are natural and non-residual alternative feed additives, which are obtained from aromatic herbs by various methods and are used as flavourings, appetite-stimulants, digestive stimulants and performance enhancers [6]. The type and level of phenolic compounds found in essential oils vary with the aromatic herbs the oils are derived from, and elicit various activities (i.e. antioxidant, antimicrobial, antifungal) [7-9]. Dietary supplementation with essential oils increases the productive capacity of poultry species by increasing the activity of digestive enzymes and eliminating pathogenic microorganisms [10,11]. Previous research has shown that carvacrol essential oils increase villus height and the villus height/crypt depth ratio, which in return improves digestive capacity [12,13]. Furthermore, rosemary oil essential oils have also been reported to increase the digestion of fat and fat-soluble vitamins by enabling the binding of bile acids to substances, and thereby, to improve the feed conversion rate [14]. Thus, it is considered that the combined use of synergistic essential oils, which are derived from different aromatic herbs and show different effects, even at low but appropriate doses, could improve animal production [15].

People have increasingly demanded poultry meat as an alternative source of animal protein [16]. Furthermore, goose meat is healthy, wholesome, and contains low cholesterol levels. Therefore, compared to other poultry species, geese are well adapted to cold climate conditions, more resistant to diseases, and do not require to be housed in costly pens. Although geese are classified as waterfowl, they are capable of living in drylands. Geese make good use of pasture plants and can be fed by grazing alone [17].

This study was aimed at the investigation of the impact of two different doses of an EO mixture on performance and intestinal histomorphology in native Turkish geese.

MATERIAL and METHODS

Ethical Approval

This study was conducted according to the approval (KAU-HADYEK/2019-118) granted by the Local Ethics Board for Experimental Animals of Kafkas University.

Animals and Treatment Design

The study was carried out at the premises of the Research Farm of Kafkas University. Sixty 1-day-old native Turkish goslings *(Anser anser)* of both sexes constituted the animal material. After hatching, the goslings were weighed, identified by unique numbers, and assigned to three groups. Each 20 animals in groups were divided into four subgroups containing five animals. The animals were homogeneously distributed among the groups by weight (32.76±0.01 g). The essential oil mixture was given to the three treatment groups at concentrations of 0%, 0.1% and 0.2%, respectively, in drinking water. During the first two weeks of the study, the animals were housed in cages under favourable environmental conditions and were fed on commercial goose starter ration containing 21.93% of crude protein (CP) and 3010 kcal/kg of metabolizable energy (ME) [18]. As from the third week, the animals were raised on the floor and acclimatized to grazing. The minimum area provided to each animal raised on the floor was 0.5 m^2. As of the third week, the animals were not only grazed but also provided with a barley meal (BM). Feed and water were provided *ad libitum*. The study was continued for ten weeks. The nutrient and chemical compositions of the starter ration, barley meal and pasture plants that the geese were fed on are presented in *Table 1*. The flora of the pasture consists of 64% Gramineae, 23% Leguminosae, and 13% other species. The treatment groups received different doses of essential oil and aromatic herb mixture containing peppermint oil, garlic oil, aniseed oil, fennel oil, cinnamon and cumin. The mixture was a commercial product supplied from Austria (BIOMIN GmbH).

Performance

Starting from the day of hatching to the day of slaughter, the animals were weighed individually on a weekly basis to determine their live weight gain. The feed intake (first two weeks concentrate feed-next eight weeks barley meal) of the animals was also calculated on a weekly basis. Feed conversion rates (FCR) were determined by calculating the ratio of feed intake to live weight gain.

Carcass Traits

Ten-week-old geese were fasted for 12 h prior to slaughter. Then, six animals, representative of the live weight of the groups, were selected from each group and were sacrificed by slaughtering the head at the occipital-atlantoaxial articulation. The birds were bled for 10-15 min and defeathered with machines. Following evisceration, the liver, gizzard and heart were weighed individually. The hot carcass weights of the unchilled carcasses were determined.

Histological Procedure

The duodenal tissue samples taken from the geese were firstly fixed in 10% formalin solution for 24 h. Then routine

Table 1. *Composition and nutrient ingredients of the feeds*

Ingredients (starter ration)		**%**
Corn, yellow		64.40
Soybean meal, 48% CP		21.46
Fish meal, 64% CP		7.00
Sunflower meal, 32% CP		4.80
Vegetable oil		0.55
Limestone		0.65
Dicalcium phosphate		0.35
DL-methionine		0.08
L-Lysine HCl		0.15
L-Threonine		0.06
Salt		0.25
Vit-Min Mix*		0.25
Nutrient Values		
Dry matter (%)		89.72
Crude protein (%)		21.93
Ca (%)		0.87
Available P (%)		0.44
Na (%)		0.16
Met+Cys (%)		0.90
Lysine (%)		1.34
Threonine (%)		0.91
Tryptophan (%)		0.26
Metabolic Energy, (kcal/kg)		3010
Chemical Analysis		**%**
Items	**Barley Meal**	**Pasture**
Dry matter (%)	88.11	94.55
Crude protein (%)	12.28	7.96
Crude fat (%)	2.18	1.65
Crude ash (%)	3.73	7.43
Crude fiber (%)	5.41	42.88
Neutral detergent fiber (%)	23.23	60.30
Acid detergent fiber (%)	6.35	48.27
Lignin (%)	0.57	9.62
Non-protein nitrogen (%)	76.41	40.09
Non-fiber carbohydrate (%)	58.59	22.67
Hemicellulose (%)	16.88	12.04
Starch (%)	55.23	-
Metabolic Energy Poultry kcal/kg	3097.05	-

** Vit-Min mix: Vit A: 10.000IU, Vit D_3: 4.000IU, Fe (iron sulfate monohydrate): 30 mg; I (calcium iodine anhydride): 1.5 mg, Co (cobalt carbonate monohydrate): 0.5 mg, Cu (copper sulfate pentahydrate): 5 mg, Mn (manganese oxide): 80 mg, Zn (zinc oxide): 80 mg, Se (selenium selenite): 0.3 mg*

tissue processing was applied and embedded in paraffin. Five-micron-thick sections were cut from the paraffin blocks and applied Mallory's modified triple staining (Triple) with a view to demonstrate the general structure of the duodenal tissue. The sections were examined with a light microscope (Olympus BX51, Japan). The crypt depths of the geese in all groups were measured using the ImageJ (LOCI, University of Wisconsin) software.

Table 2. *Effects of EO on performance parameters of geese (Mean±SEM; n=20)*

Periods	**EO Groups**	**LWG (g)**	**FI (g)**	**FCR**
0-2. weeks	0.0%	33.91±0.98	49.93±0.27	1.48±0.04
	0.1%	33.18±1.53	49.59±0.24	1.50±0.07
	0.2%	32.32±0.17	49.53±0.28	1.53±0.01
P		0.584	0.531	0.680
2-4. weeks	0.0%	51.51±0.79	121.33±0.28	2.36±0.04
	0.1%	51.16±0.26	121.53±0.39	2.38±0.02
	0.2%	51.90±0.86	121.88±0.38	2.35±0.04
P		0.755	0.559	0.688
4-6. weeks	0.0%	45.47±0.78	171.05±0.48	3.76±0.06
	0.1%	45.81±0.67	168.55±0.57	3.68±0.05
	0.2%	49.49±1.17	169.18±0.45	3.67±0.05
P		0.787	0.403	0.423
6-8. weeks	0.0%	49.10±0.69	201.24±0.50	4.27±0.09
	0.1%	50.03±0.71	201.21±0.45	4.13±0.11
	0.2%	48.55±0.66	201.58±0.61	4.08±0.11
P		0.342	0.858	0.424
8-10. weeks	0.0%	48.87±0.32	228.60±0.34	4.66±0.03
	0.1%	47.52±0.85	228.61±0.64	4.82±0.08
	0.2%	48.05±0.70	228.28±0.44	4.75±0.07
P		0.385	0.376	0.279
0-10. weeks	0.0%	45.36±0.25	154.53±0.11	3.40±0.02
	0.1%	45.09±0.40	153.90±0.08	3.41±0.03
	0.2%	45.59±0.17	154.09±0.05	3.29±0.04
P		0.534	0.059	0.635

***EO:** Essential oil; **LWG:** Live weight gain; **FI:** Feed intake; **FCR:** Feed conversion rate. **SEM:** Standart error of the mean*

Statistical Analysis

The differences between the groups for performance, carcass traits, and histological parameters were analysed using the Statistical Package (SPSS portable PASW 18) software by one-way analysis of variance (ANOVA). The paired comparison of the data was performed with Duncan's test. The significance level was accepted as $P<0.05$.

RESULTS

The effects of the EO mixture on growth performance are shown in *Table 2*. The results obtained in the present study demonstrated that the EO mixture had no effect on live weight gain, feed intake and feed conversion rate ($P>0.05$).

Values pertaining to the slaughter and carcass traits of the study groups are presented in *Table 3*. Accordingly, it was

Table 3. *Effects of EO on carcass traits of geese (Mean±SEM; n=6)*

EO Groups	Slaughter Weight (g)	Carcass Yield (%)	Liver (g)	Gizzard (g)	Heart (g)
0.0%	3263.4±22.64	71.66±0.51	161.60±2.25	187.60±10.69	24.80±1.36
0.1%	3236.2±52.66	71.22±0.33	161.60±2.94	178.40±6.55	25.80±1.74
0.2%	3173.3±34.84	71.38±0.30	161.40±2.11	170.80±4.90	25.00±1.97
P	0.279	0.724	0.998	0.343	0.909

EO: *Essential oil;* ***SEM:*** *Standart error of the mean*

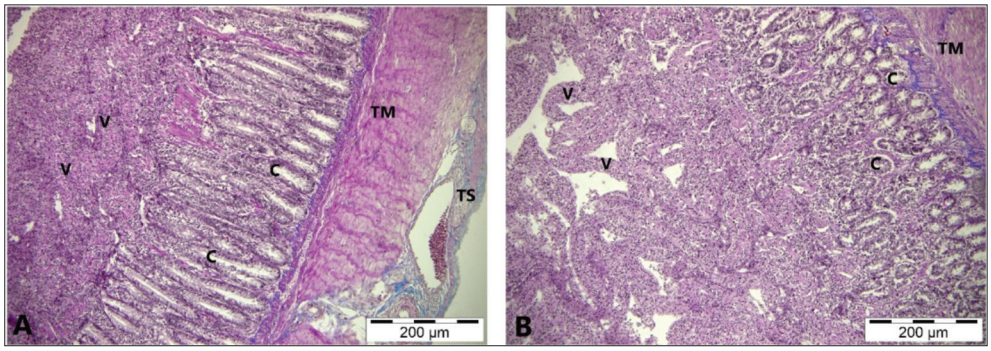

Fig 1. Goose duodenum tissue. **A:** 0.1% EO group, **B:** 0.2% EO group. **V:** Intestinal villi, **C:** Crypts, **TM:** Tunica muscularis, **TS:** Tunica serosa; Triple staining

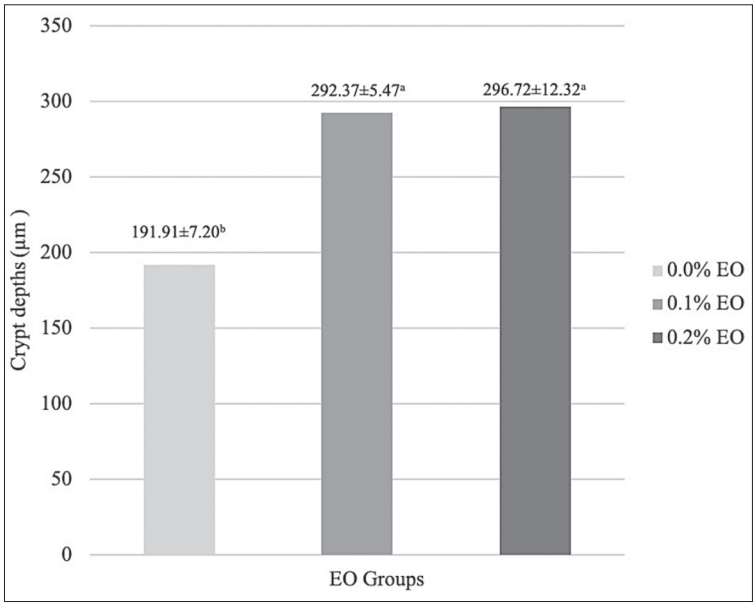

Fig 2. Effects of EO on duodenal crypt depths (µm), (Mean±SEM; n=6); [a,b] Values in the same column with no common superscript differ significantly (P<0.05); **EO:** Essential oil; **SEM:** Standart error of the mean

determined that the groups did not differ for the weight at slaughter and carcass yield of the animals (P>0.05). Similarly, no significant difference was determined between the groups for liver, gizzard and heart weights (P>0.05). The average weight of the liver, which is one of the most valuable products of geese, was determined to be 161.60 g, 161.60 g and 161.40 g, respectively, in the groups that were given 0%, 0.1% and 0.2% EO.

In all groups, the duodenal tissue of the geese was observed to be composed of the innermost tunica mucosa, tunica muscularis consist of smooth muscle cells and the outermost tunica serosa consist of connective tissue layers. The tunica mucosa formed the intestinal villi and crypts *(Fig. 1, Fig. 2)*.

The statistical analysis of the intestinal crypt depths of all groups demonstrated that the 0.0% EO group significantly differed from the 0.1% and 0.2% EO groups (P<0.05).

DISCUSSION

No statistically significant difference existed between the EO treatment groups (0.0%, 0.1% and 0.2%) for the live weight gain values between weeks 0-10 of the study. Generally, it is considered that herbal extracts produce favourable results in poultry. However, there are very few studies conducted on the use of aromatic herbs in geese. In these limited number of studies, it has been suggested that, in geese, performance values are not affected by supplementation with aromatic herbs [19,20]. The results of

the present study are in agreement with these previous poultry studies reporting unaffected performance [21-23]. Researchers have also reported that improved environmental conditions and reduced pathogen burden decrease the effects expected from the use of essential oils. Jamroz et al.[24] contrary to reported that 100 mg of an essential oil mixture containing carvacrol, cinnamaldehyde and capsicum increased live weight parameters in broiler chickens. This increase was attributed to cinnamaldehyde, which is the main active substance of cinnamon, increasing the secretion of pancreatic and intestinal enzymes, and capsicum increasing the production of hepatic enzymes, bile, pancreatic enzymes and intestinal lipase, resulting in an increased absorption of nutrients [24,25]. The results of the present study do not agree with some literature reports [26-28]. Some studies suggest that essential oil supplementation shows adverse effects on the performance of poultry [29]. Differences between the results of the present study and previous research have been attributed to different essential oil components having been used and tested, the essential oils found in the composition of mixtures interacting with each other, and EOs having been used at different levels. Furthermore, it is considered that geographical conditions, the timing of harvest, as well as animal- and environment-related factors may also contribute to discrepancies in study results.

Throughout the study period, no significant difference was observed between the groups for weekly feed intake and feed conversion rate values. In previous studies conducted by Baowei et al.[19] and Yaman et al.[20] in geese, it was reported that neither feed intake nor feed conversion rate was affected. Similarly, while Fascina et al.[30] indicated that essential oil mixtures had no effect on growth performance, feed intake and feed conversion rate, Nobakht and Mehmannavaz [31] reported that different fat resources did not cause any change in average feed intake values in broiler chickens. Furthermore, Khaksar et al.[32] reported that thymol essential oil had no effect on feed intake and feed conversion rate in quails. These findings are in agreement with previous studies, which were conducted by Mathlouthi et al.[33], Küçükyılmaz et al.[34], and suggested that essential oil mixtures did not affect feed intake and feed conversion rate.

The effects of essential oils arise from the active molecules found in their structure. The results of the present study are similar to those reported by Lee et al.[35] and Günal et al.[36], who suggested that herbal extracts do not affect feed intake in broiler chickens, but contradict with the report of Tekeli et al.[37], who suggested that herbal extracts increase feed intake in broiler chickens. Jamroz et al.[24] reported that the supplementation of maize- and wheat-based rations with an essential oil mixture containing carvacrol, cinnamon oil and black pepper oil increased the feed conversion rate of broiler chickens by 4%. On the other hand, Al-Kasie [38] indicated that supplementation with an essential oil mixture containing carvacrol and cinnamon oil decreased the feed conversion rate, when given at a dose of 200 ppm ($P<0.05$).

The end-study carcass traits and visceral organ weights of the groups are presented in *Table 3*. Accordingly, no statistically significant difference was observed between the groups for slaughter weight, carcass yield and heart, liver and gizzard weights ($P>0.05$).

The findings obtained in the present study comply with those reported by Muhl and Liebert [39], who suggested that the supplementation of broiler grower rations with phytogenic extracts (5% carvacrol, 3% cinnamaldehyde and 2% *Capsicum oleoresin)* had no effect on carcass yield, and also agree with the report of Şimsek et al.[40], who indicated that mixtures of carvacrol, clove oil and aniseed oil do not have any effect on carcass yield and characteristics in broiler chickens. Furthermore, similar to the present study, upon investigating the effects of different protein sources in geese, Şahin et al.[41] reported to have not detected any difference between the study groups for carcass traits. The similarities between the results of these different studies are attributed to the similarity of the management conditions the animals were exposed to.

The results obtained for carcass yields in the present study differ not only from those reported by Fascina et al.[30] who indicated a significant increase in carcass yield with the supplementation of broiler rations with phytogenic extracts, but also from those reported by Al-Kasie et al.[38] who pointed out to significantly increased carcass yields with the supplementation of broiler rations with essential oil mixtures containing carvacrol and cinnamon oil ($P<0.01$).

Literature reports have been published, which suggest that herbal feed additives improve slaughter and carcass traits [40,42]. Researchers attribute this positive effect to the appetizing, enzyme-secretagogue and antimicrobial activity of essential oils [43].

The results of the present study are similar to those reported by Criste et al.[44] for gizzard and hearth weights, by Kim et al.[45] for liver weight, by Yeganeparast et al.[22] for hearth weights, and by Salehifar et al.[46] for liver and gizzard weights. Çelik and Şahin [47] determined that an essential oil mixture, which was administered in drinking water and contained peppermint oil, carvacrol, cade oil and rosemary oil did not affect heart, liver and gizzard weights. The visceral organ weight parameters determined in the present study differ from the results of some other previous studies conducted on essential oil supplementation [42,48].

Low crypt depth is one of the main indicators of intestinal health. Peng et al.[49] reported that the supplementation of broiler rations with oregano essential oil reduced the

depth of intestinal crypts, and thereby, increased growth performance. Some other studies have reported that essential oil supplementation does not affect duodenal crypt depth in broiler chickens [50,51]. In agreement with the present study, Yang et al.[52] reported that cinnamon oil, when supplemented at a high dose (800 mg/kg), increased duodenal crypt depth, but had no effect on performance parameters. In fact, increased crypt depth was observed not to show any adverse effect on performance parameters in geese.

In conclusion, it has been determined that, in geese, supplementation with different doses of essential oil mixtures increases intestinal crypt depth, but this increase does not affect live weight gain. In view of the limited number of studies in geese, it is considered that further studies need to be carried out in poultry, and particularly in geese, such that different doses of various essential oils are investigated in different slaughtered age.

Conflict of Interest

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

Author Contributions

Experimental design, feeding trial and article writing; MÖ and TŞ, Performance parameters calculation; ÖK, Histological analysis; EKS, Statistical Analysis; SAI and TK, Interpretation and editing of results; MAY.

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Research Article

Determination of Oxidative Stress Index and Total Sialic Acid in Cattle Infested with *Hypoderma* spp.

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Abstract

Hypodermosis is a parasitic disease, which is caused by larvae of *Hypoderma lineatum* and *Hypoderma bovis* flies in cattle and causes damage on the skin. It causes economic losses due to decrease in meat and milk yield, growth deficiency and being of no use in leather processing as a result of perforation of the leather. The aim of this study was to define the oxidative-antioxidative capacity and total sialic acid levels in cattle with hypodermosis. A total of 90 cattle was used in the study, among those, 30 with hypodermosis and 60 antibody-negative healthy bovine sera were used according to ELISA test results to investigate the biochemical parameters. Biochemical analysis included total oxidant capacity (TOC), total antioxidant capacity (TAC), oxidative stress index (OSI), and total sialic acid (TSA). TAC value was found to be statistically significantly lower ($P<0.01$), TOC, OSI and TSA values were found to be higher ($P<0.05$) in cattle with hypodermosis compared to the control group. It was concluded that hypodermosis caused significant changes in sialic acid concentration and oxidative-antioxidative capacity, which are indicators of acute phase response in cattle with hypodermosis. It is thought that these obtained findings can particularly contribute to determination and diagnosis of inflammation intensity in disease.

Keywords: *Cattle, Hypodermosis, Oxidative stress, Total sialic acid*

Hypoderma spp. İle Enfeste Sığırlarda Oksidatif Stres İndeksi ve Total Siyalik Asit Düzeyinin Belirlenmesi

Öz

Hypodermosis, sığırlarda *Hypoderma lineatum* ve *Hypoderma bovis* tipi sineklerin larvaları tarafından oluşturulan ve deride hasara neden olan paraziter bir hastalıktır. Hayvanların et ve süt veriminde azalma, büyüme eksikliği ve derinin delinmesi sonucunda deri işlemede kullanılamaması gibi ekonomik kayıplara neden olur. Çalışmanın amacı *Hypoderma* ile enfeste sığırlarda oksidatif-antioksidatif kapasite ve total siyalik asit düzeyinin belirlenmesidir. Çalışmada, ELISA test sonuçlarına göre, 60 antikor negatif ve 30 hypodermosisli olmak üzere toplam 90 adet sığır serumu kullanıldı. Biyokimyasal olarak total oksidan kapasite (TOK), total antioksidan kapasite (TAK), oksidatif stres indeksi (OSİ) ve total siyalik asit (TSA) analizi yapıldı. Kontrol grubu ile hypodermosisli sığırlar karşılaştırıldığında istatistiksel olarak TOK, OSİ ve TSA düzeyinin arttığı ($P<0.05$), TAK düzeyinin ise azaldığı ($P<0.01$) belirlendi. Sonuç olarak, hypodermosisin akut faz yanıtın bir göstergesi olan siyalik asit konsantrasyonunda ve oksidatif-antioksidatif kapasitede önemli değişikliklere neden olduğu belirlendi. Elde edilen bu bulguların özellikle hastalıktaki inflamasyon şiddetinin belirlenmesine ve teşhisine katkıda bulunabileceği düşünülmektedir.

Anahtar sözcükler: *Sığır, Hypodermosis, Oksidatif stres, Total siyalik asit*

INTRODUCTION

Hypodermosis is a parasitic disease, which is created by larvae of *Hypoderma lineatum* and *Hypoderma bovis* type flies in cattle and causes damage on the skin [1,2]. It causes economic losses due to decrease in meat and milk yield of animals, growth deficiency, stress-related immune depression, and being of no use in leather processing as a result of perforation of the leather [3,4]. Glycoproteins, glycolipids, polysaccharides, and acylated derivatives of neuraminic acid formed by condensation of sialic acid, mannosamine and phosphoenolpyruvate, are involved in

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the structure of mucoproteins [5,6]. While 85% of the sialic acid is bound to the protein part of the glycoconjugates and about 15% to the lipid, a very small amount is free. Total sialic acid (TSA) is the total of free protein and lipid-bound sialic acid [7]. Sialic acid has functions such as regulation of cellular communication, determining recognition in host-pathogen interactions [8]. It has been demonstrated that sialic acid can be an important indicator for early and accurate diagnosis, rational treatment and effective prophylactic measures in both infectious and noninfectious diseases [7,9].

While there is a balance between oxidants and antioxidants in normal organism; stress, chronic disease and infections in organism stimulate immune system and tissue damage, causing inflammatory reactions due to increase in free radical production [10].

When free radicals exceed the amount that is required for the body, they cause an oxidative damage by interacting with the molecules such as lipids, carbohydrates, proteins and nucleic acids [10,11]. The system, which functions in order to prevent the damage created by the free radicals, is defined as the antioxidant system [12]. In studies, it is reported that total oxidant capacity (TOC) and total antioxidant capacity (TAC) or oxidative stress index (OSI) change and can be used as a non-invasive marker in local and/or systemic inflammation or infections [13-16]. The aim of this study was to investigate TSA, TOC and TAC and OSI status in cattle infested by hypoderma.

MATERIAL and METHODS

The research work was carried out with the approval of the Institutional Ethics Committee of Kafkas University, Faculty of Veterinary Medicine (KAU 2014/011). 90 cattle of Brown Swiss breed, aged between 2 and 5 years, were used in the study. These animals were obtained from dairy farms in Kars, Turkey. These farms were visited during September and November of 2016. The animals in control group were received an antiparasitic drug (Ivomec F; Novakim, Turkey, 200 µg/kg/bw) to remove any presence of internal parasite before use. Routine clinical examinations (fever, number of breaths, pulse, native examination of the feces, etc.) were carried out for each animal. Blood samples obtained from *jugular veins* of animals were collected into plain tubes, centrifuged at 3000 rpm for 15 min and obtained sera were stored at -20°C until analyzed. The presence of Hypoderma antibodies in serum samples was investigated by an ELISA kit (IDEXX Bovine Hypodermosis Antibody Test Kit-P06110). The test was performed according to manufacturer's instructions, and results were measured spectrophotometrically (Epoch, Biotek, USA). According to ELISA test results 60 hypodermosis infested and 30 antibody-negative healthy bovine sera were used to investigate the biochemical parameters.

The levels of TOC and TAC were measured using commercially available Rel Assay diagnostic kits (Gaziantep, Turkey) via microplate reader (Epoch, BioTek, USA). Briefly, hydrogen peroxide and trolox were used as standards to calculate for TOC and TAC, respectively. OSI which is the indicator of the degree of oxidative stress was calculated using (Arbitrary Unit) = [TOC (µmol H_2O_2 equivalent/L)/10xTAC (mmol Trolox equivalent/L)] formula [17]. TSA concentrations were determined according to the spectrophotometric method developed by Sydow [18] (Epoch, Biotek, USA).

Statistical Analysis

SPSS [19] for Windows 20.0 was used for the statistical analyses. The distribution of the data obtained from the groups were shown as normal distribution according to the Kolmogorov-Smirnov test. Therefore, Student's t-test was then used to compare the differences of the values observed in group with infected with those observed in control group.

RESULTS

Clinical examination showed that the general status of both control and infested animals were in good health and no parasite eggs were found in the feces of the animals.

In this study, there were significant increases in the concentrations of TOC ($P<0.05$), OSI ($P<0.05$) and TSA ($P<0.05$) levels in the *Hypoderma spp.* infested group compared to the control group. However, TAC ($P<0.01$) values were significantly lower in infested group than in the control group *(Table 1)*.

DISCUSSION

Hypodermosis is a parasitic disease, which causes economic losses such as affect weight gain, welfare, bovine immune defense mechanisms and the leather industry [20,21]. Larval secretions of *Hypoderma* spp. are comprised of three main components (hypodermins A, B and C) with potent anti-inflammatory and immunosuppressive effects in primo-

Table 1. *Total oxidant & antioxidant capacity and total sialic acid levels in control and Hypoderma spp. infested groups*

Parameters	Control	Infested	P
TAC (mmol Trolox Eq/L)	1.18±0.08	0.92±0.05	P<0.01
TOC (µmol H_2O_2 Eq/L)	29.64±1.46	38.02±2.82	P<0.05
OSI (Arbitrary Unit)	3.03±0.33	4.89±0.52	P<0.05
TSA (mg/dL)	64.65±2.00	70.94±1.61	P<0.05

infested cattle. In addition, the migrating fly larvae trigger serious allergic reactions and inflammation in cattle. Acute phase response occurs depending on cellular and humoral response during migration of the parasite larva in tissues of animals [22-26] and concentration of sialic acid of which function is found in acute phase response increases depending on the damage occurred in cell membrane during infestation [27]. TSA values increase considerably in infectious, tumoral and metabolic diseases [9]. It has been reported that serum TSA value increases in some diseases such as theileriosis, anaplasmosis [28], echinococcosis [9], foot and mouth disease [29], and leptospirosis [30]. In this study, it was also specified that TSA value increased considerably in cattle with hypodermosis than healthy animals. Increased TSA value in cattle with hypodermosis can be explained by tissue damage which was caused during the migrating fly larva.

Although there are many methods for determining oxidative stress, these methods are complex and expensive methods that require long time and effort, and measuring the oxidant/antioxidant molecules individually allows only the evaluation of the molecule being measured. Therefore, it has been reported that TAC and/or TOC measurement is more beneficial rather than measuring oxidants or antioxidant individiually in order to specify oxidant/antioxidant balance [31,32].

It has been reported that oxidative stress developes in viral, bacterial and parasitic diseases such as foot and mouth disease [29], brucellosis [15], ascaridiosis [33] and sheep-poxvirus infection [34]. it has been stated that parasitic infections cause damage in cells and tissues depending on increase of the free radicals in host cells [35]. The studies have reported in cattle infested with hypoderma, it has been stated that oxidative stress developes [36,37]. In this study, it was also specified that TOC value increased and TAC value decreased in infested cattle in which oxidative stress developed in parallel to the study conducted. The reason of increase in TOC can be explained by the fact that parasitic infections cause free radical related damage in cells and tissues of the host [35]. It was also specified in the study that OSI value increased considerably compared to the control group. OSI, which is defined as the ratio of TOC value to TAC value, is an indicator of oxidative stress value and OSI value reflects oxidative condition in animals infected with hypodermosis.

In conclusion, significant changes in sialic acid concentration and oxidative-antioxidative capacity, which are an indicator of acute phase response, occur in in cattle with hypodermosis. It is thought that these obtained findings can particularly contribute to determination and diagnosis of inflammation intensity in disease.

Author Contributions

This work was carried out in collaboration between all authors. KB, OM and GTT: Designed the experimental procedures. KB, OM and NA: Conducted the research work. OM, NA: Helped in laboratory analysis. OM, GGT, KB: Prepared tables, revised and submitted the manuscript. All authors read and approved the final manuscript.

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Research Article

Protective Effects of Adenovirus-Mediated Overexpression of Heat Shock Protein 70 (HSP70) in Rat Liver Cells Against Oxidative Stress

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Abstract

This study was aimed to study the protective effects of Adenovirus-mediated overexpression of heat shock protein 70 (HSP70) in rat liver cells against oxidative stress. Cultured Buffalo Rat Liver cells (BRL-3A) cells were divided into six groups. Groups SH and CH were transfected with Ad-CMV-HSP70, groups SN and CN were transfected with Ad-CMV-Null, and groups SC and CC were treated under the same conditions without adenovirus for 48 h. Groups SH, SN and SC were treated with 500 μM H_2O_2 for 3 h, and groups CH, CN and CC were treated under the same conditions without H_2O_2. The following parameters were determined for cells of each group: proliferation rate, lactic dehydrogenase (LDH) leakage rate, catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) enzyme activities, and glutathione (GSH) and protein carbonyl contents. In addition, the HSP70 mRNA abundance and HSP70 protein expression levels were examined using quantitative real-time PCR (qRT-PCR) and western blotting, respectively. The result shows that compared with cells of group SC, cells of group SH had a higher proliferation rate ($P<0.01$), CAT activity ($P<0.01$), GSH content ($P>0.05$), SOD activity ($P>0.05$), and protein carbonyl content ($P<0.01$), a lower LDH leakage rate ($P<0.01$) and GSH-Px activity ($P\leq0.05$), and higher HSP70 mRNA abundance ($P<0.01$) and HSP70 protein expression level ($P<0.01$). In summary, Adenovirus-mediated overexpression of HSP70 has certain protective effects on rat liver cells against H_2O_2-induced oxidative stress.

Keywords: *HSP70, BRL-3A, Oxidative stress, Adenovirus vector, Cold stress*

Sıçan Karaciğer Hücrelerinde Isı Şok Protein 70 (HSP70)'in Adenovirüs Aracılı Aşırı Ekspresyonunun Oksidatif Strese Karşı Koruyucu Etkileri

Öz

Bu çalışmada, sıçan karaciğer hücrelerinde ısı şok protein 70 (HSP70)'in Adenovirüs aracılı aşırı ekspresyonunun oksidatif strese karşı koruyucu etkilerinin araştırılması amaçlamıştır. Kültürlenmiş Buffalo Rat Karaciğer hücreleri (BRL-3A) altı gruba ayrıldı. SH ve CH grupları Ad-CMV-HSP70 ile, SN ve CN grupları Ad-CMV-Null ile transfekte edildi. SC ve CC grupları ise 48 saat boyunca aynı koşullar altında adenovirüs olmadan tedavi edildi. SH, SN ve SC gruplarına 3 saat boyunca 500 μM H_2O_2 uygulandı ve CH, CN ve CC grupları H_2O_2 olmadan aynı koşullara maruz bırakıldı. Her bir gruptaki hücreler için aşağıdaki parametreler belirlendi: proliferasyon hızı, laktik dehidrogenaz (LDH) sızıntı oranı, katalaz (CAT), glutatyon peroksidaz (GSH-Px) ve süperoksit dismutaz (SOD) enzim aktiviteleri, glutatyon (GSH) ve protein karbonil içeriği. Ek olarak, HSP70 mRNA yoğunluğu ve HSP70 protein ekspresyon seviyeleri, sırasıyla kantitatif gerçek zamanlı PCR (qRT-PCR) ve western blot yöntemi kullanılarak incelendi. Sonuçlar, SC grubundaki hücrelerle karşılaştırıldığında, SH grubundaki hücrelerin daha yüksek proliferasyon oranına ($P<0.01$), CAT aktivitesine ($P<0.01$), GSH içeriğine ($P>0.05$), SOD aktivitesine ($P>0.05$), ve protein karbonil içeriğine ($P<0.01$), daha düşük bir LDH salınım oranı ($P<0.01$) ve GSH-Px aktivitesine (P0.05) ve daha yüksek HSP70 mRNA varlığı ($P<0.01$) ve HSP70 protein ekspresyon seviyesine ($P<0.01$) sahip olduğunu gösterdi. Özetle, HSP70'in Adenovirüs aracılı aşırı ekspresyonu, sıçan karaciğer hücreleri üzerinde H_2O_2'nin neden olduğu oksidatif strese karşı belirgin koruyucu etkilere sahiptir.

Anahtar sözcükler: *HSP70, BRL-3A, Oksidatif stres, Adenovirüs vektörü, Soğuk stresi*

INTRODUCTION

Since Sies published Oxidative Stress in 1985, oxidative stress has increasingly been used as an indicator in research into biochemical systems for the influence of oxidative processes, particularly in the biomedical field [1]. Research over the last 30 years has shown that oxidative stress is closely involved in the majority of diseases and in aging of organisms. Many factors are known to cause oxidative stress, including electricity, cold, heat, and atmospheric

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pollution. Thus, many stresses such as cold stress, heat stress, and transport stress are closely related to oxidative stress. Oxidative stress provides a convenient entry point for solving the damage caused to animals by adverse stresses in livestock production.

Several studies have shown that compensatory changes occur in the antioxidant defense system of mammals exposed to low temperatures [2]. Once an animal is exposed to a cold environment, the protein structure suffers more severe oxidative damage in the liver than in the kidney or heart [3]. Therefore, the injury mechanism of cold stress and anti-stress pathways in animals could be investigated through the study of oxidative stress in liver cells. The stress protein HSP70 is one of the major molecular chaperones, which helps to maintain cellular protein homeostasis and improve the tolerance of cells to stressors to maintain the normal physiological functions of cells. Our previous work showed that liver HSP70 rapidly increases in organisms under cold stress, helping the animal resist the external stimuli [4]. In the present study, we plan to transfect BRL-3A cultured *in vitro* with recombinant adenovirus carrying HSP70 to observe the proliferation of HSP70-expressing hepatocytes under H_2O_2-induced oxidative stress. Condition and degree of oxidative damage. The Results permitted the evaluation of the protective effect of recombinant adenovirus HSP70 transfection on rat liver cells against oxidative stress.

Newborn animals generally have weak ability to keep warm, wintering in northern alpine regions, and the impact of extreme weather on animals. The cold stress involved in these three aspects is gradually attracting scientific researchers' attention. Production practice and previous scientific research results have shown that cold stress can cause a decline in animal production performance, reduce animal disease resistance, and cause death in severe cases. Therefore, how to solve the problem of eagerly improving the ability of animals to resist cold stress in production practice has become an urgent issue in the field of animal husbandry research. In the process of cold stress in animals, the liver is damaged due to oxidative stress. This experiment studies animal cold stress from the perspective of liver cell oxidative stress, which will help to study the role of HSP70 in animal anti-cold stress, open up new ideas for cold stress research, reveal the mechanism of cold stress for the future and lay the foundation for anti-stress approach.

MATERIAL and METHODS

Materials

Competent *Escherichia coli* DH5α cells were obtained from the Animal Stress Biology Laboratory of Heilongjiang Bayi Agricultural University. The BRL-3A cell line was provided by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The adenovirus expression system (Adxsi Vector System) was purchased from SinoGenoMax (Beijing, China). Commercial kits for assaying WST-1 cell proliferation and cell toxicity, total GSH, total SOD activity, CAT, GSH-Px and BCA protein content (enhanced) were purchased from Beyotime Institute of Biotechnology (Haimei, China). Kits for protein carbonyl content assay and LDH assay were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The HSP70 monoclonal antibody was purchased from Abcam and the GAPDH monoclonal antibody and HRP-labeled goat anti-mouse IgG purchased from Santa Cruz (Santa Cruz, CA, USA). Other imported or domestic reagents were all analytical grade.

Cell Culture

BRL-3A cells frozen in liquid nitrogen were taken out, placed in a 37°C water bath environment and continuously shaken to allow rapid melting. The freezing tube was thoroughly sterilized with 75% ethanol, and the cell suspension was transferred aseptically to a 15 mL centrifuge tube. The cells were washed with 10 mL of cell culture broth (10% FBS) and centrifuged at 500x g for 5 min. The supernatant was removed and the cell pellet was washed again, re-suspended in 10 mL of cell recovery solution (20% FBS), transferred to a 75 cm^2 cell culture flask and incubated in an incubator (37°C, 5% CO_2). Thereafter, the cell culture broth (10% FBS) was replaced on a daily basis. When the cell density exceeded 1×10^6 mL^{-1}, the cell culture was digested with 0.25% trypsin (w/w) and then passaged into several 96-well plates, 6-well plates and 25 cm^2 cell culture flasks for routine incubation for 2-3 d. All experiments were performed on cells in the logarithmic growth phase and in good condition.

Experimental Grouping

The BRL-3A cells were randomly divided into six groups. These include three non-stressed groups: recombinant adenovirus Ad-CMV-HSP70 transfected (CH), empty vector Ad-CMV-Null transfected (CN) and no virus (CC), as well as three H_2O_2-stressed groups: recombinant adenovirus Ad-CMV-HSP70 transfected (SH), empty vector Ad-CMV-Null transfected (SN), and no virus (SC). Each group was set with 3-5 parallel replicates.

Recombinant Adenovirus Vector Construction and Target Cell Transfection

Recombinant adenovirus vector construction: The HSP70 gene sequence was amplified from Wistar rat spleen tissues using RT-PCR and cloned into vector pMD18-T to construct the recombinant cloning plasmid pMD18-T-HSP70. Nucleotide sequencing showed that the obtained sequence has a total length of 2269 bp and contains the complete CDS of HSP70. This is 100% homologous to the amino acid encoded by the same gene of rats (accession number: NP_114177) published in Genbank. The cloned HSP70 reading frame was sub-cloned into the pShuttle-CMV shuttle vector to obtain the recombinant plasmid

pShuttle-CMV-HSP70. The expression cassette of HSP70 was cut from the recombinant shuttle vector using I-Ceu I and I-Sce I, and ligated into to the pAdxsi vector carrying the adenovirus backbone, and identified via XhoI restriction enzyme analysis and sequencing. The recombinant adenovirus backbone plasmid was digested with PacI for linearization, and transfected into 293 (R) cell lines for virus packaging.

Target cell transfection: The BRL-3A cells were harvested at the exponential stage under good growth conditions and washed once with phosphate buffered saline (PBS). The cells of groups SH and CH were transfected with cell culture containing 1×10^7 pfu/mL Ad-CMV-HSP70 (MOI = 20); groups SN and CN were transfected with cell culture containing an equal amount of Ad-CMV-Null; and groups SC and CC were supplemented with an equal volume of virus-free cell culture. All six cell cultures were returned to the incubator (37°C, 5% CO_2). The plates were slightly shaken for 10 sec at 15 min intervals. Cultural broth was supplemented after 6 h. The total incubation period was 48 h.

Oxidative Stress Model

The oxidative treatment solution was prepared by diluting 30% H_2O_2 solution with cell culture broth to a final concentration of 500 µM. The culture broth was removed from all six groups of cell cultures, and the cell pellets washed once using PBS. The oxidative treatment solution was added to groups of SH, SN and SC, and an equal volume of H_2O_2-free culture medium was added to groups CH, CN and CC. All cell cultures were incubated for 3 h and then harvested for subsequent analyses.

WST-1 Cell Viability Assay

The WST-1 solution (10 µL) was added to cells from the six groups in 96-well plates and then incubated in an incubator for 1 h. The solution was thoroughly mixed before absorbance measurement using a microplate reader (at a detection wavelength of 450 nm; and a reference wavelength 630 nm). Each measurement was repeated three times.

Lactate Dehydrogenase Activity Assay

Six groups of BRL-3A cell cultures and the corresponding lysates were harvested from 25 cm^2 cell flasks for lactate dehydrogenase (LDH) activity assay using a commercial kit. The LDH leakage rate (%) = culture LDH activity/(culture LDH activity + cell LDH activity) × 100%.

Catalase Activity Assay

Six groups of BRL-3A cell culture lysates were harvested from 25 cm^2 cell culture flasks, and then centrifuged at 12.000x g (4°C) for 10 min. Catalase (CAT) activity was determined in the supernatant using a commercial kit, and the remaining material was used for protein content assay according to the BCA method.

Glutathione Peroxidase Activity Assay

Six groups of BRL-3A cell culture lysates were harvested from 25 cm^2 cell culture flasks, and then centrifuged at 12.000x g (4°C) for 10 min. Glutathione peroxidase (GSH-Px) activity was determined in the supernatant using a commercial kit, and the remaining material was used for protein content assay according to the BCA method.

Glutathione Content Assay

For the glutathione (GSH) content assay, the 25 cm^2 cell flasks of the six groups of BRL-3A cells were washed with 5 mL of PBS for 30 s. After the removal of PBS, cells were aseptically scratched and transferred to 1.5 mL micro-centrifuge tubes. The cell culture was centrifuged at 1.000x g for 3 min, and the supernatant removed. Thereafter, following the manufacturer's instructions, the GSH content of the deposit was assayed for each group of BRL-3A cell cultures.

Superoxide Dismutase Activity Assay

Six groups of BRL-3A cell culture lysates were harvested from the 25 cm^2 cell culture flasks, and then centrifuged at 12.000 g (4°C) for 10 min. The supernatant was used for the superoxide dismutase (SOD) activity assay using a commercial kit, and the remaining material was used for protein content assay according to the BCA method.

Protein Carbonyl Content Assay

The BRL-3A cell cultures in 25 cm^2 cell bottles were washed twice with pre-chilled PBS. After the removal of the PBS, the cells were scraped and transferred to 1.5 mL centrifuge tubes. The harvested cells were centrifuged and the supernatant discarded. The cells were resuspended in pre-chilled normal saline and subjected to ultrasonication (40% amplitude) in an ice bath for 5 s x 12, with 30 sec intervals. The protein carboxyl content was analyzed in 0.45 mL of homogenate using a commercial assay kit, and the remaining material was used for protein content assay following the BCA method.

qReal-time PCR

Total RNA was extracted using the Trizol method and cDNA was synthesized as a PCR template under standard conditions required by the reverse transcription kit. Specific primers were designed to amplify the HSP70-encoding gene using Primierprimer5.0. The upstream primer was 5'-GCTCGAGTCCTACGCCTTCAATA-3', and the downstream primer was 5'-TCCTGGCACTTGTCCAGCAC-3'. The amplification product was 105 bp in length. Specific primers for an internal reference gene β-actin were: upstream 5'-TCACCAACTGGGACG-3' and downstream 5'-GCATACAGGGACAACA-3'. The fluorescent quantitative real-time PCR (qRT-PCR) amplification was performed in three steps: 95°C denaturation for 2 min; 40 cycles of 94°C denaturation for 10 sec, 58°C annealing for 30 sec, and 72°C extension for 30 sec; and a final extension at 95°C for

30 min. Fluorescent signals were collected at the end of each cycle, and the melting solution curve analysis was performed after all cycles were finished. Results from qRT-PCR assays were used for relative quantitative calculation with the software package Line-Gene K provided with the qRT-PCR machine, following the maximum second derivative method.

Western Blot Analysis

Cells were harvested and total cellular protein was extracted from the lysate. Ten micrograms of protein sample were loaded onto SDS-PAGE containing 10% polyacrylamide, and then transferred to nitrocellulose membranes using the wet transfer method. The membrane was rinsed in PBST for 5 min three times, and then incubated in 5% non-fat milk solution for 1 h. The membrane was rinsed with PBST for 10 min three times and then cut into two pieces, which contained the HSP70 band and internal reference GAPDH band, respectively. These two membranes were incubated in 1:2000 and 1:5000 primary antibody solutions, respectively, at 37°C for 1 h. Thereafter, the membranes were rinsed with PBST for 10 min three times, and then incubated in 1:5000 secondary antibody solution at room temperature for 1 h. The membranes were then rinsed with PBST for 10 min three times. The membranes were then developed, fixed and exposed to X-ray film, which were photographed. The photographs were analyzed using Image J and statistical data were processed with Excel.

Statistical Analysis

Analysis of variance (ANOVA) was performed using SPSS 17.0. All measurements are presented as mean values ± standard deviation (X±Sx).

RESULTS

Ad-CMV-HSP70 Construction

According to XhoI restriction enzyme analysis and sequencing analysis, the recombinant adenoviral vector Ad-CMV-HSP70 was successfully constructed. The viral particles were purified through CsCl density-gradient centrifugation and the titer of recombinant adenoviral was calculated based on the absorbance (A260 and A260/A280) and TCID50. The titer of recombinant adenoviral reached 1.0×10^{11}pfu/mL, which was suitable for further transfection experiments.

Cell Viability

Of the three non-stressed groups, the average OD values followed the order of CC>CN>CH, with significant differences among the groups (P<0.01) *(Fig. 1)*. For the three stressed groups, the average OD values were highest in the SN group followed by SH and SC, with significant differences among the groups (P<0.01). The average OD values were lower in the stressed group than in the non-stressed group (P<0.01), suggesting that the cell proliferation rate was decreased. The largest decrease was found in SC (0.491), followed by SN (0.107) and SH (0.007) *(Fig. 1)*.

LDH Leakage Rate Variation

The LDH leakage rate and cellular content were both higher in cells of the groups with oxidative stress compared with those of the groups without stress *(Table 1)*. Of these, the LDH leakage rates of groups SN and SC were significantly higher than those of the corresponding groups without stress (P<0.01). The LDH leakage rate was higher in the group SH compared with the group CH (P<0.01), but the LDH cellular contents have no significant difference (P>0.05) between the two groups. The LDH leakage rates of groups CH and CN were significantly higher than that of group CC

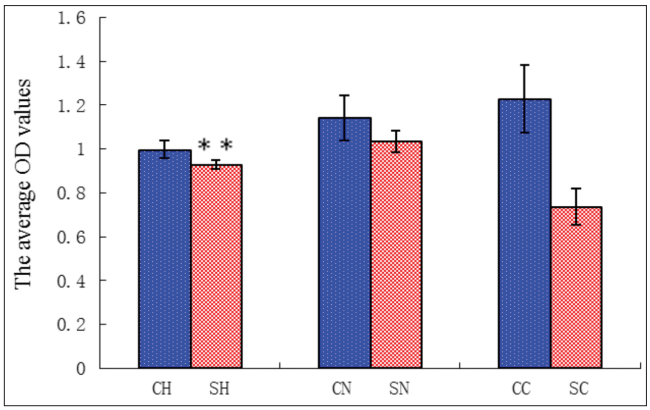

Fig 1. BRL-3A cell proliferation rate indicated by the average OD values. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

Table 1. *Lactate dehydrogenase activity of BRL-3A cells*

Group	Leakage Rate U/L	Cellular Content U/gprot	Total U
CH	896.13±58.69 [a]	1.84±0.05 [a]	48.71±1.42 [a]
SH	941.58±14.36 [b]	1.86±0.01 [a]	49.23±0.15 [a]
CN	784.53±46.26 [c]	1.97±0.11 [ab]	51.70±2.77 [a]
SN	1049.46±68.86 [d]	2.04±0.02 [b]	54.19±0.74 [b]
CC	756.54±45.63 [c]	2.00±0.22 [ab]	52.28±5.59 [ab]
SC	1413.94±23.20 [e]	2.27±0.01 [c]	60.76±0.21 [c]

Different superscript letters in the same column represent statistically significant differences (P<0.01)

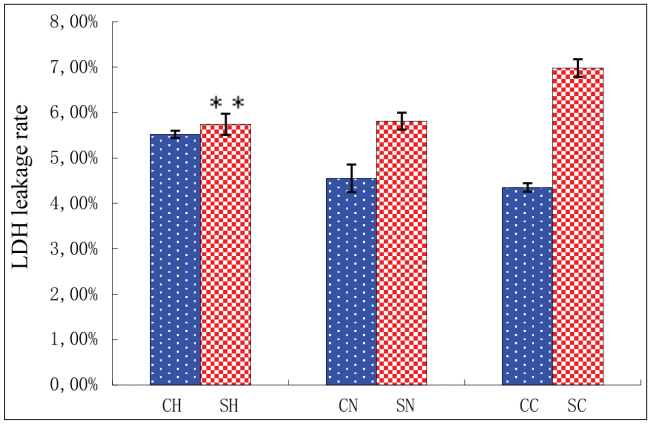

Fig 2. LDH leakage rate in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

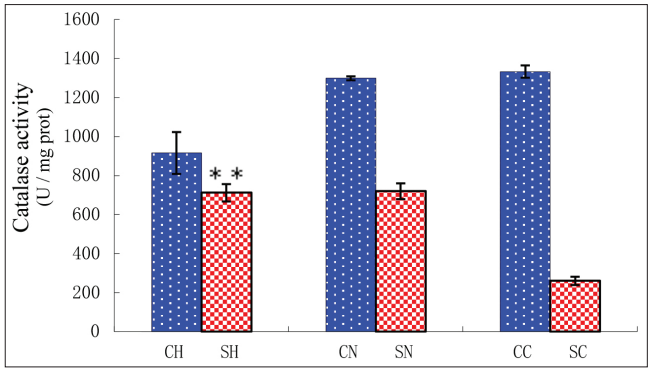

Fig 3. Catalase activity in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

(P<0.01), but significantly lower in groups SH and SN compared with group SC (P<0.01). There were no significant differences in LDH leakage rate between groups SH and SN (P>0.05) *(Fig. 2)*.

Catalase Activity

The CAT activity was significantly lower in cells of the stressed groups compared with those of the non-stressed groups (P<0.01) *(Fig. 3)*. Of these, the CH group had lower CAT activity than groups CN (P≤0.05) and CC (P<0.01), whereas the SH group had higher CAT activity than the SC group (P<0.01), with no significant difference from that of the SN group (P>0.05) *(Fig. 3)*.

Glutathione Peroxidase Activity

The GSH-Px activity was lower in cells of the groups subjected to oxidative stress compared with those of the non-stressed groups. However, such difference was only significant between the corresponding virus-free groups (P≤0.05) *(Fig. 4)*. For the non-stressed groups, the CH group had significantly lower GSH-Px activity than groups CN (P<0.01) and CC (P<0.01). Of the three stressed groups, SH had a lower GSH-Px activity than SN (P<0.01) and SC (P≤005) *(Fig. 4)*.

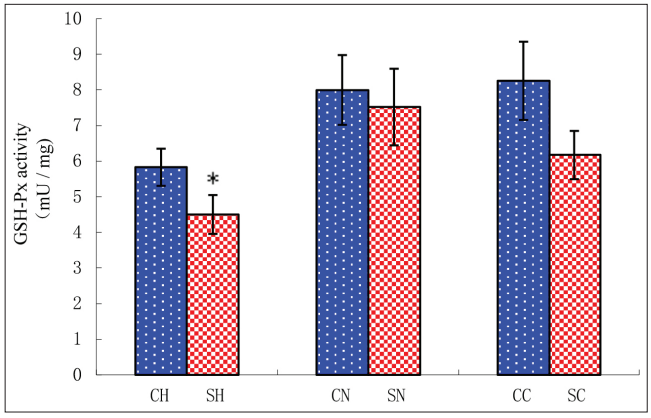

Fig 4. GSH-Px activity in BRL-3A cells of different groups. Results are expressed as mean ± SD. *P≤0.05 for comparison with group SC

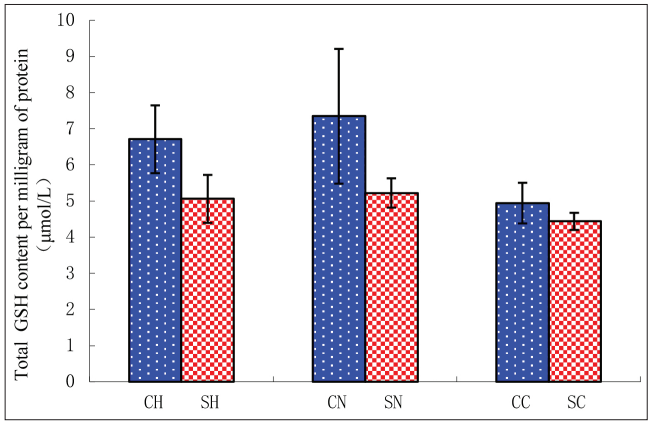

Fig 5. GSH content in BRL-3A cells of different groups. Results are expressed as mean ± SD. P>0.05 for group SH versus group SC

Glutathione Content

The GSH content was significantly higher in the CH group compared with the CC group (P≤0.05), whereas there were no significant differences between the GSH contents of groups SH and SC (P>0.05) *(Fig. 5)*. The GSH content was significantly reduced in the two groups with adenovirus transfection upon oxidative stress (P≤0.05), but no significant decline was found in the virus-free groups after oxidative stress (P>0.05) *(Fig. 5)*.

Superoxide Dismutase Activity

The SOD activity was significantly lower in cells of the CH group compared with those of the CN group (P≤0.05), but significantly higher than the CC group (P<0.01) *(Fig. 6)*. The SOD activity was significantly lower in cells of the SH group than that of the SN group (P<0.01), with no significant difference with the SC group (P>0.05) *(Fig. 6)*.

Protein Carbonyl Content

The protein carbonyl content was significantly higher in cells of the stressed group compared with the non-stressed group (P<0.01) *(Fig. 7)*. There were no substantial

differences in protein carbonyl content amongst the non-stressed groups (P>0.05), but the protein carbonyl content was significantly higher in cells of the SH group compared with groups SN and SC (P<0.01) *(Fig. 7)*.

HSP70 mRNA Abundance

The stressed groups had significantly higher HSP70 mRNA expression levels than non-stressed groups (P<0.01), and the SH group had significantly higher HSP70 mRNA expression levels than groups SN and SC (P < 0.01) *(Fig. 8)*.

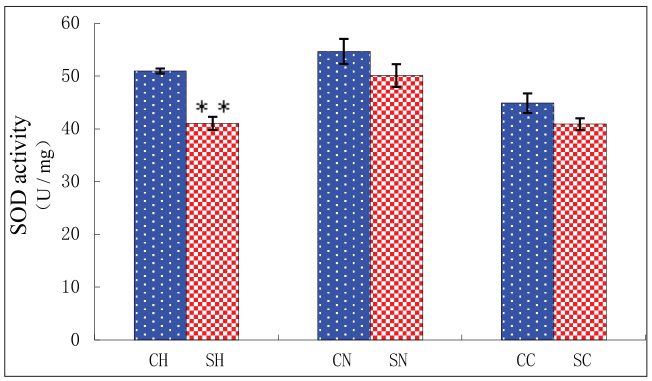

Fig 6. SOD activity in BRL-3A cells of different groups. Results are expressed as mean ± SD. *P≤0.05 for comparison with group SN

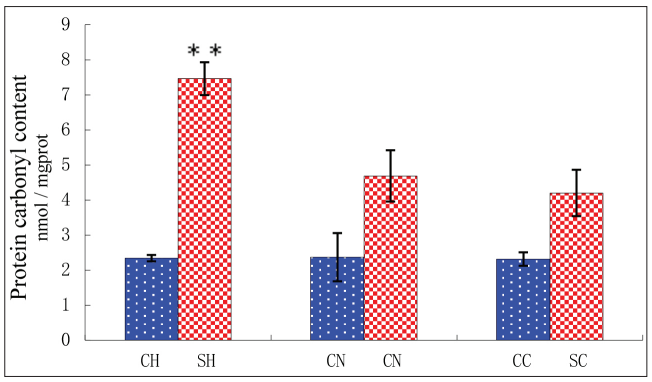

Fig 7. Protein carbonyl content in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

Hsp 70 Protein Expression Level

As shown in *Fig. 9* and *Fig. 10*, cells of the stressed groups had significantly higher HSP70 protein expression levels than the non-stressed groups (P<0.01), and the SH group had significantly higher HSP70 protein expression level than groups SN and SC (P<0.01)

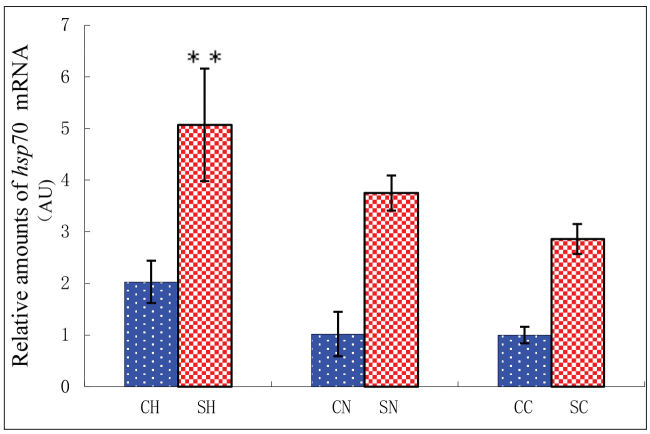

Fig 8. HSP70 mRNA abundance in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC group SC

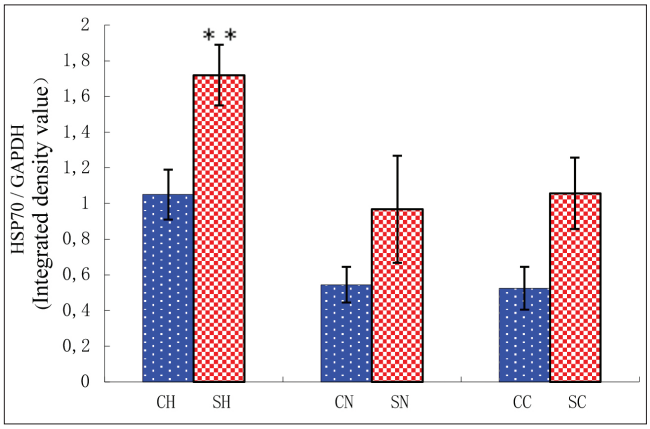

Fig 10. Hsp 70 protein expression levels in BRL-3A cells of different groups. Results are expressed as mean ± SD. ** P<0.01 for comparison with group SC

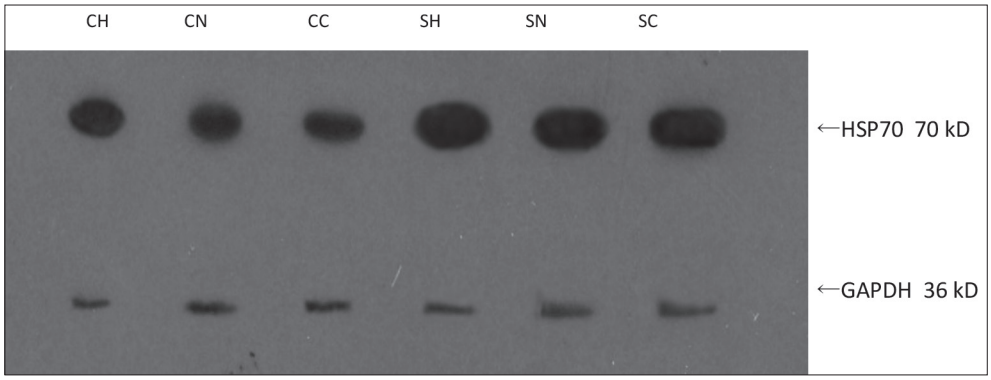

Fig 9. Detection of HSP70 by western blotting. The position of the molecular weight markers is indicated

DISCUSSION

The cell viability results from this study showed that average OD values in cells of the three stressed groups were significantly lower than those of non-stressed groups (P<0.01). This indicated that the 3 h oxidative stress induced by 500 μM H_2O_2 on BRL-3A cells caused decreases in cell proliferation rates or damage to mitochondria. Of the three non-stressed groups, the average OD value was highest for the cells of the CC group, followed by CN and CH (P<0.01). We speculated that both Ad-CMV-HSP70 and Ad-CMV-Null (MOI = 20) cause cell damage or death to a certain degree. The WST-1 assay also showed that the average OD values of Ad-CMV-HSP70 were lower than those of Ad-CMV-Null; therefore, the HSP70-containing adenovirus vector likely caused greater damage to cells under non-stressed conditions. This was confirmed by the subsequent LDH assay. However, the changes in the average OD value of the non-stressed group and the stressed group showed that, compared with cells transfected with empty vector or virus-free cells, transfection of BRL-3A cells carrying HSP70 is advantageous in resisting H_2O_2-induced damage, as indicated by smallest decreases in their average OD values (P<0.01).

Lactic dehydrogenase release is an important signal of apoptosis. When the cytomembrane is damaged, LDH will be released from *in vitro* cell cultures into the culture medium, and the release amount reflects the degree of damage to cells caused by physical factors or drug treatment [5]. In the present study, all stressed groups had substantially higher LDH leakage rates than non-stressed groups, suggesting that the 3 h 500 μM H_2O_2-stress caused damage to BRL-3A cell membranes, leading to the increased LDH leakage rate. In addition, our measurements of LDH leakage were probably less than the actual LDH leakage from cells, because the acidity of the H_2O_2 solution could cause partial inactivation of the LDH leakage. In 2018, Wu et al. found that HSP70 reduced the leakage of LDH in rats pretreated with hyperbaric oxygen [6]. Our results showed that overexpression of HSP70 reduced LDH leakage. Our results showed that the LDH leakage rate was significantly lower in the SH group than in SC (P<0.01), despite a lack of significant difference between groups SH and SN (P>0.05). These results indicated that the adenovirus-mediated HSP70 transfection of BRL-3A cells had a protective function, but it remains uncertain whether such a function was the result of exogenous introduction of HSP70.

Catalase is an important component of the cellular ROS scavenging system. It has an important role in maintaining the balance of intracellular ROS metabolism, and catalyzes the production of H_2O and O_2 from H_2O_2 [7]. Hence, the H_2O_2 stimulation to BRL-3A cells could cause intracellular CAT consumption, accounting for the substantial reduction of CAT activity in cells of the stressed groups compared with the non-stressed groups (P<0.01). In addition, CAT activity was substantially lower in cells of the group CH compared with other non-stressed groups (P≤0.05), possibly due to the overexpression of HSP70. In Cheng and Dimitrovskas' experiments, a similar negative correlation was also observed between HSP70 and CAT [8,9]. We speculate that CAT and HSP70 are both transiently expressed proteins, thus the rapid expression of HSP70 potentially imposed a competitive inhibition effect on the expression of CAT during the stress process.

Glutathione peroxidase is an important enzyme that occurs widely in organisms and catalyzes the decomposition of H_2O_2. It specifically catalyzes H_2O_2 reduction to protect the structural and functional integrity of the cell membrane. In this study, the H_2O_2-stressed group had substantially lower GSH-Px activity than the corresponding non-stressed group. This was probably because the oxidation of oxygen free radicals led to carbonyl production from amino acid residues of the GSH-Px protein, further changing the secondary structure of GSH-Px and reducing its α-helix percentage [10]. Our results showed that regardless of the stress, the lowest GSH-Px activity occurred in cells transfected with Ad-CMV-HSP70. These results suggested that the expression of HSP70 reduced GSH-Px activity, consistent with previous findings. In 2019, Liu et al.[11] found that GSH-Px activity is negatively correlated with HSP70 expression. The low-molecular-antioxidant GSH is the substrate of GSH-Px. GSH can eliminate oxygen free radicals and H_2O_2, stabilize sulfhydryl enzymes, prevent oxidative damage, and combine with toxic substances to accelerate their catabolism [12]. In the present study, the two adenovirus transfection groups had substantial decreases in GSH content upon H_2O_2 stress (P≤0.05), indicating that H_2O_2 caused a certain degree of oxidative damage to cells. However, no significant differences were observed between GSH contents of the SH group and other stressed groups in the present study. It was likely that HSP70 first reduced the GSH-Px activity, and subsequently inhibited GSH production.

Superoxide dismutase can eliminate superoxide anion radicals to protect cells from damage. It plays a vital role in maintaining the balance of oxidation and antioxidation in organisms [13]. Our results showed that all BRL-3A cells of the stressed groups had lower SOD activity than the non-stressed groups (P≤0.05), consistent with previous findings. For example, Guesmi found that H_2O_2 treatment of rats can significantly reduce the SOD activity in the testis of rats [14]. It was likely that H_2O_2 or OH-• enhanced the inactivation effect of H_2O_2 on SOD and increased the protein carbonyl content of the enzyme, further reducing the enzymatic activity. In addition, the relationship between HSP70 and SOD activity is inconclusive. In the present study, we found that SOD activity was substantially lower in cells of the SH group compared with the SN group (P<0.01). This could be related to the exogenous introduction of HSP70, but the detailed mechanism(s) remain unclear.

The protein carbonyl content is a sensitive indicator of protein oxidative damage. A high protein carbonyl content indicates that the protein suffers larger oxidative damage and that the cell and living organism has suffered a greater degree of oxidative stress [15]. In the cold-exposure animal test, the protein carbonyl contents of different varieties of rat livers were significantly increased compared with the control after short- or long-term cold exposure [16]. In our study, the protein carbonyl content was significantly higher in cells of the stressed groups compared with the non-stressed groups, suggesting that H_2O_2 caused oxidative damage to BRL-3A cells. In addition, the protein carbonyl content was higher in the SH group compared with the other stressed groups. According to the theory that protein carbonyl content correlates with the extent of oxidative damage, the damage to cells of the SH group should be the largest, which is consistent with results from other assays of cell proliferation, LDH leakage rate and antioxidant enzyme activities. It was likely that the cells of group SH contained more HSP70 than other groups, and that the overexpressed HSP70 functioned as the 'death squads' that were first attacked by oxidative stress. The sacrifice of overexpressed HSP70 protected other proteins against the attack of oxidative stress. In addition, some researchers consider that HSP70 becomes the target of carbonylation through 4-hydroxy azelaic acid [17]. Presently, the protective mechanisms of HSP70 against oxidative damage to cellular proteins remain unclear. Further study is needed to produce sufficient experimental evidence to confirm our speculation.

Comprehensive comparisons of relevant indicators between the cells of groups SH and SC showed that adenovirus-mediated HSP70 transfection imposed a certain protective effect on rat liver cells against oxidative stress. However, it remains unclear whether this protective effect is entirely caused by the exogenous introduction of inducible HSP70, as various indicators showed no significant differences between groups SH and SN. There are several potential reasons. Firstly, the adenovirus vector itself imposed a certain degree of stress to BRL-3A cells, and this stress can initiate the self-protection of cells, thus allowing transfected cells more advantages than virus-free cells in response to subsequent oxidative stress. Secondly, although inducible HSP70 can protect cells from stress damage, it needs to synergize with other members of the Hsp family for better performance. In this study, exogenous introduction of inducible HSP70 increased the expression level of inducible HSP70. However, the contents of synergized proteins would show no corresponding increases. Hence, the protective function inducible HSP70 could not come into play completely. Third, the BRL-3A cells can produce endogenous inducible HSP70 under stress. Thus, a large amount of endogenous HSP70 can be expressed initially, even in the absence of exogenous introduction of HSP70 under stress. Consequently, the HSP70 level of stressed BRL-3A cells transfected with Ad-CMV-HSP70 was not higher than that of cells free of the virus. Fourth, the adenoviral vector is associated with transient transfection. The exogenously introduced HSP70 is only available for transcription but not replication. In addition, HSP70 mRNA has a short half-life in normal cells and is easily degraded; thus, high levels of translation and expression of exogenous HSP70 cannot be guaranteed.

In summary, HSP70 is likely to be the most important intermediary substance in cells response to oxidative stress. An increasing number of reports have shown that HSP70 is negatively correlated with oxidative stress [18]. However, our results suggest that the adenovirus-mediated HSP70 transfection only has a certain protective effect on rat liver cells against oxidative stress. Further study is needed to explore the anti-oxidant mechanism in liver cells associated with HSP70.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

All authors read and approved the final version of the article. Designed the study: Designed the study: Guo Jingru. Conducted the analyses and wrote the first draft of the article: Hu Huijie, Liu Hongrui, Yuan Jianbin. Tables and results: Ji Hong, Zhang Xu.

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Research Article

Complete Genome Sequencing of *Mycoplasma bovis* Type Strain Ningxia-1 and Systematic Bioinformatic Characterization for Housekeeping-related Genes

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Abstract

Mycoplasma bovis is a major pathogen leading to bovine respiratory disease syndrome. The worldwide prevalence of this pathogen has caused enormous economic losses in the beef industry. Substantial efforts have been made to identify and characterize the surface proteins of *M. bovis*. However, little is known regarding experimentally proved housekeeping genes, or the distribution and the number of motifs within these genes in *M. bovis*. We used Picbio SMRT technology of next-generation sequencing for *M. bovis* Ningxia-1 isolation and applied different tools (Mega X, STRING v11.0, TMHMM v2.0, MOTIF) for bioinformatics analysis. The present study compared *M. bovis* Ningxia-1 strain with another ten *M. bovis* strains with sequenced whole genome and identified 24 housekeeping genes in each strain. The phylogenetic tree indicates a close relationship between *M. bovis* Ningxia-1 with NM2012 based on BLAST results of these genes. Within the 24 housekeeping genes in *M. bovis* Ningxia-1 stain, 3/24 (12.5%) of the genes have the potential to be used as internal control genes, 2 sets of proteins have interactions which have been proved under experimental and database conditions. MetG is a putative transmembrane protein, while others are predicted to be located outside of the membrane. Additionally, there are 6 common motifs distributed among 7 of the proteins (29.17%). Our bioinformatic analysis is intended to provide new and complementary data in mining and making comparisons of housekeeping genes through *M. bovis* type strain Ningxia-1 sequencing.

Keywords: *Mycoplasma bovis, Next-generation sequencing, Housekeeping gene, Bioinformatic analysis*

Mycoplasma bovis Ningxia-1 Suşunun Tüm Genom Sekanslaması ve Housekeeping İlişkili Genlerin Sistematik Biyoinformatik Karakterizasyonu

Öz

Mycoplasma bovis, sığır solunum hastalığı sendromuna yol açan önemli bir patojendir. Bu patojenin dünya çapında yaygınlığı, sığır eti endüstrisinde büyük ekonomik kayıplara neden olmuştur. *M. bovis*'in yüzey proteinlerini tanımlamak ve karakterize etmek için önemli çabalar sarf edilmiştir. Bununla birlikte, *M. bovis*'te deneysel olarak kanıtlanmış housekeeping genleri veya bu genler içindeki motiflerin dağılımı ve sayısı hakkında çok az şey bilinmektedir. *M. bovis* Ningxia-1 izolasyonu için yeni nesil sekanslama Picbio SMRT teknolojisini kullandık ve biyoinformatik analiz için farklı araçlar (Mega X, STRING v11.0, TMHMM v2.0, MOTIF) uyguladık. Bu çalışma ile *M. bovis* Ningxia-1 suşu, sekanslanmış tam genomlu başka bir *M. bovis* suşu ile karşılaştırıldı ve her suşta 24 housekeeping geni tanımladı. Filogenetik ağaç, bu genlerin BLAST sonuçlarına dayanarak M. bovis Ningxia-1 ile NM2012 arasında yakın bir ilişki olduğunu gösterdi. *M. bovis* Ningxia-1 boyasındaki 24 housekeeping geni içinde, genlerin 3/24'ü (%12.5) internal kontrol genleri olarak kullanılma potansiyeline sahiptir, proteinlerin 2 seti deneysel ve veritabanı koşullarında kanıtlanmış etkileşim göstermektedir. MetG varsayılan bir transmembran protein olmasına rağmen, diğerlerinin membranın dışında olduğu tahmin edilmektedir. Ek olarak, 7 proteine (%29.17) dağılmış 6 yaygın motif bulunmaktadır. Biyoinformatik analizimiz, veri madenciliğinde yeni ve tamamlayıcı veriler ortaya koymuş ve *M. bovis* tipi Ningxia-1 dizileme yoluyla temel genlerin karşılaştırmalarını yapmayı sağlamıştır.

Anahtar sözcükler: *Mycoplasma bovis, Yeni nesil dizileme, Housekeeping gen, Biyoinformatik analiz*

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INTRODUCTION

Mycoplasma bovis is an important pathogen causing a variety of disease syndromes in cattle. *M. bovis* can affect a large variety of tissues and organs [1], causing mastitis [2], pneumonia [3], arthritis [4], keratoconjunctivitis [5], otitis media [6], meningitis [7], or reproductive diseases [8]. At present, the pathogen is prevalent worldwide, which causes considerable economic losses to feedlot cattle, dairy and veal calf industries [9], with hugely detrimental impacts on animal welfare [10]. *M. bovis* was reported as resistant to anti-microbial drugs including the β-lactams, polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin [11], due to the lack of a cell wall and absence of a murein/peptidoglycan layer. It is known that *M. bovis* can be shed in the infected cattle for months or even years [12]. To date, no vaccines are commercially available, however, the macrolides, modified macrolides, tetracyclines, aminoglycosides, chloramphenicols, pleuromutilins and fluoroquinolones can treat *M. bovis* infection as potential medications [10]. Draxxin (Tulathromycin) is the only approved drug for treatment for *M. bovis* and the normal duration is 10-14 days of antibiotic therapy [13].

The first complete genome of *M. bovis* PG45 was published in 2010, and genomes of two other Chinese isolates (Hubei-1 and HB0801) were reported in 2011 and 2012. With the development of sequencing technology, next-generation sequencing (NGS) for microbes using Picbio SMRT technology is rapidly becoming a common method for characterization of the whole genome of all living species, allowing for much higher levels of detail [14]. It is an invaluable tool for comparative genomic studies through the analysis of single nucleotide polymorphisms (SNPs) and gene-based comparative methods [15]. To date, the genome of 11 *M. bovis* has been fully sequenced and are available from NCBI Genbank. We collected these 11 different *M. bovis* genomes together with their biosample information and compared these strains using 24 housekeeping genes presented by a phylogenetic tree.

The genome of *M. bovis* Ningxia-1 contains a single circular chromosome of 1,033,629 bp, with a GC content of 29.3% [16]. Housekeeping genes are a group of constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions [17]. Additionally, housekeeping genes are instrumental for calibration in many computational applications and genomic studies and are used widely as internal controls for experiments [18,19]. To date, less information is known about *M. bovis* housekeeping genes. Thus, we collected all published housekeeping genes from the Pubmed database to compare and analyze these genes using bioinformatic approaches. Systematic analysis-based software, such as Mega X, STRING v11.0, TMHMM v2.0, MOTIF, were used to identify relationships of molecular evolution on gene sequence alignment, protein-protein interactions, membrane distribution and all motifs respectively in these housekeeping genes. Our urpose of these sequence-based methods was to identify various housekeeping genes in *M. bovis* Ningxia-1 strain and to define these 24 genes using different KEGG pathways, cellular location, motif distribution and the potential clinical detection markers. We identified 3/24 (12.5%) potential genes which could be used as clinical detection markers for further molecular experimental study. 4/24 (16.67%) of the proteins had protein-protein interactions under experimental and databases conditions, 23/24 (95.8%) proteins are an outside membrane protein, and 6 common motifs were distributed in 7/24 (29.17%) proteins.

MATERIAL and METHODS

Strain and Culture

The Ningxia-1 strain was isolated from the lung of an infected calf and stored at the College of Agriculture, Ningxia University, China. The strain was cultured in a pleuropneumonia-like organisms (PPLO) broth (1 g glucose, 21 g PPLO, 100 mL 25% yeast, 2 g sodium pyruvate, 200 mL calf serum (BD, Waltham, MA, USA), 0.1g of 80,000 IU/ mL penicillin-G, and 4.5 mL 0.4% phenol red). Type 2 water (Millipore, Burlington, MA) was added to make 1 L of broth. The inoculation amount was v/v 1:10 at 37°C for 3 days on an orbital shaker.

Genomic DNA Preparation

The cultured *M. bovis* was harvested from 1 L of broth by centrifugation at 12.000×g under 4°C for 30 min, the supernatant was discarded and pelleted cells were sent to the GeneDenovo Guangzhou, China for genome sequencing. Genomic DNA was extracted using commercial kits according to the manufacturer's instructions. The DNA quality was detected using Qubit (Thermo Fisher Scientific, Waltham, MA) and Nanodrop (Thermo Fisher Scientific, Waltham, MA) accordingly.

Sequencing

Qualified genomic DNA was fragmented with G-tubes (Covaris) and end-repaired to prepare SMRTbell DNA template libraries (with fragment size of >10 Kb selected by bluepippin system) according to the manufacturer's specifications (PacBio, Menlo Park, CA). A total of library quality was detected by Qubit and average fragment size was estimated on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Single Molecule Real Time (SMRT) sequencing was performed on the Pacific Biosciences RSII sequencer (PacBio, Menlo Park, CA) according to standard protocols (MagBead Standard Seq v2 loading, 1 × 180 min movie) using the P4/C2 chemistry [20].

De Novo Genome Assembly

Continuous long reads were attained from three SMRT

sequencing runs. Reads longer than 500 bp with a quality value over 0.75 were merged together into a single dataset. Next, the hierarchical genome-assembly process (HGAP) pipeline was used to correct for random errors in the long seed reads (seed length threshold 6 Kb) by aligning shorter reads from the same library against them [21]. The resulting corrected, preassembled reads were used for de novo assembly using Celera Assembler with an overlap-layout-consensus (OLC) strategy [22]. Since SMRT sequencing features very little variations of the quality throughout the reads, no quality values were used during the assembly [23]. To validate the quality of the assembly and determine the final genome sequence, the Quiver consensus algorithm was used [21]. Finally, the ends of the assembled sequence were trimmed to have the genome circularized. The depth of final sequencing is 1262 fold.

Extraction and Mining for Housekeeping Genes

Mycoplasma bovis strain PG45 (CP002188.1) and *M. bovis* Ningxia-1 24 housekeeping genes were selected from Pubmed (US National Library of Medicine and the National Institutes of Health) for STRING, trans-membrane and motif analysis. The flowchart is shown in *Fig 1*. Literature published between 2010 and 2018 was scanned using the following search terms: *M. bovis* and housekeeping genes. A list of 7 published articles was retrieved *(https://www.ncbi.nlm.nih.gov/pubmed)*. Articles were reviewed and analyzed for diagnostic methods by amplification and molecular methods, as well as for clinical diagnosis markers.

Genomic Prediction and Evolutionary Position

The reference sequence NZ_CP023663.1 was derived from CP023663. Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline (released 2013). Information about the Pipeline can be found at *https://www.ncbi.nlm.nih.gov/genome/annotation_prok/*. Meanwhile, we identified 11 *M. bovis* and one *Mycoplasma agalactiae (M. agalactiae)* genomes with whole genome sequencing. Twenty-four selected housekeeping genes were aligned by MUSCLE [25]. DNA sequences of 11 orthologous mycoplasma species and one *M. agalactiae* were aligned using a maximum likelihood (ML) method with Mega X *(http://www.megasoftware.net)* [25]. The following parameters were used: 1000 replications for bootstrap analysis, "Tamura and Nei model" for the substitution model, "use all site" for the proportion of gaps/missing data treatment, "Nearest-Neighbor-Interchange (NNI)" for ML heuristic method, and "BIONJ" for starting tree(s) [25]. EvolView is a comprehensive tool for tree visualization and annotation after obtained the original phylogenetic tree for Mega [26].

BLAST Analysis

A DNA sequence comparison tool BLAST *(http://www.ncbi.nlm.nih.gov/blast/blast.cgi)* was using to compare the similarity of 24 genes in *M. bovis* Ningxia-1 strain and *M. agalactiae*. Nucleotide of 24 genes in *M. bovis* Ningxia-1 were placed as the query sequence and the DNA genome sequence of *M. agalactiae* was to deposited into the subject sequence box. More dissimilar sequences (discontiguous

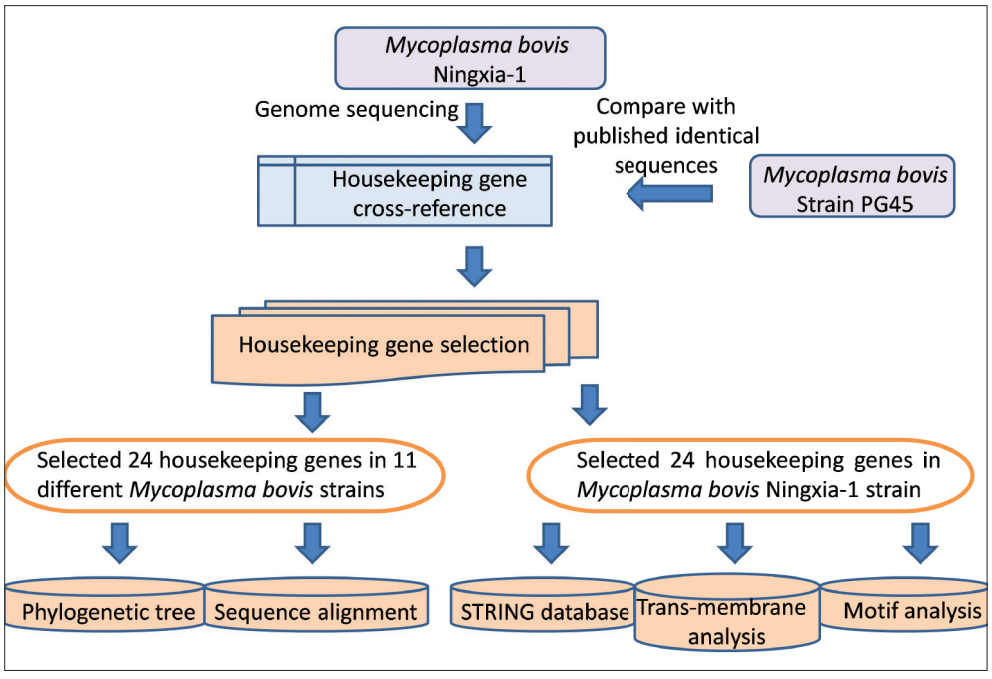


Fig 1. A systematic workflow of selecting and evaluating housekeeping genes in *M. bovis*. To compare essential housekeeping genes after the next generation sequencing of *M. bovis* Ningxia-1 isolate. *M. bovis* was searched for housekeeping genes studies in NCBI database. For each housekeeping gene collected, the PMID of the corresponding peer-reviewed articles was recorded, comparing with *M. bovis* PG45. Gene information was then identified from the NCBI Gene website, and the protein sequence was extracted for further analysis

Table 1. *The list of 11 M. bovis strain selected bioinformation in this study*

No	*Mycoplasma bovis* Strain	Isolation Source	Isolate Location	Time
1	PG45	Bovine mastitis	USA	1965
2	Ningxia-1	Lung of a beef calf (10 d after birth)	Pengyang, Ningxia, China	2013
3	Hubei-1	Lung of a calf	Hubei, China	2008
4	HB0801	Lesioned lung of an infected beef	Yingcheng, Hubei, China	2008
5	NM 2012	Synovial fluid	China:Inner Mongolia	2012
6	CQ-w70	Lung	China:Yunyang County, ChongQing Municipality	2009
7	08M	The lung of a calf with pneumonia	China	2008
8	JF4278	mastitic milk	Switzerland	2008
9	HB0801-115	Lesioned lung of an infected beef cattle, *in vitro* subculture	Yingcheng city in Hubei province, China	2012
10	HB0801-150	Lesioned lung of an infected beef cattle, *in vitro* subculture	Yingcheng city in Hubei province, China	2012
11	HB0801-180	Lesioned lung of an infected beef cattle, *in vitro* subculture	Yingcheng city in Hubei province, China	2012

Table 2. *Genome comparison in 11 selected M. bovis strains*

No.	Name	Species	INSDC	Size	GC%	Protein	rRna	tRna	Other RNA	Gene	Pseudogene
1	PG45	*Mycoplasma bovis*	CP002188.1	1,003,404	29.3	779	6	34	3	870	48
2	Hubei-1	*Mycoplasma bovis*	CP002513.1	948,121	29.3	731	4	34	3	813	41
3	HB0801	*Mycoplasma bovis*	CP002058.1	991,702	29.3	764	6	34	3	845	38
4	CQ-W70	*Mycoplasma bovis*	CP005933.1	948,516	29.3	740	4	34	3	813	32
5	NM 2012	*Mycoplasma bovis*	CP011348.1	990,348	29.3	763	6	34	3	843	37
6	08M	*Mycoplasma bovis*	CP019639.1	1,016,753	29.3	770	6	34	3	867	54
7	Ningxia-1	*Mycoplasma bovis*	CP023663.1	1,033,629	29.3	750	6	34	3	887	94
8	JF4278	*Mycoplasma bovis*	LT578453.1	1,038,531	29.3	787	6	34	3	894	64
9	HB0801-P115	*Mycoplasma bovis*	CP007589.1	977,322	29.3	738	6	34	3	834	53
10	HB0801-P150	*Mycoplasma bovis*	CP007590.1	977,304	29.3	714	6	34	3	835	78
11	HB0801-P180	*Mycoplasma bovis*	CP007591.1	977,257	29.3	694	6	34	3	835	98

megablast) were selected by a BLAST algorithm.

STRING Analysis

In order to predict interaction networks between the 24 housekeeping genes in *M. bovis* Ningxia-1 strain, the annotated genome was downloaded from the Genebank database *(https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP023663.1)*. The protein sequences of these genes were stored in a FASTA format document and then input into the latest version STRING 11.0 *(https://string-db.org/)*. The *M. bovis* PG45 organism was selected. For generating the figures, a confidence cutoff of 0.4 was used. Under evidence view, a network map was downloaded. To identify different genes involved in KEGG/MAPK pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database *(https://www.kegg.jp/kegg-bin/show_organism?menu_type=pathway_maps&org=mbv)* was used basing on enrichment function. To identify the experiment conformed genes among these 24, we chose confidence view which indicating the strength of the data support with highest confidence score of 0.9 (experiments and databases condition was chosen for analysis).

Transmembrane Analysis

To determine transmembrane probability for each housekeeping gene, their protein sequences were pasted to TMHMM Server v. 2.0 *(http://www.cbs.dtu.dk/services/TMHMM/)*. The library was set up in a FASTA format (Supplement data) and analyzed using standard settings to determine transmembrane helices in the 24 proteins.

Motif Analysis

To further expand our analysis and sort the housekeeping genes based on their expression of functional domains, the 24 housekeeping genes protein sequences were queried against the Motif Search Library *(https://www.genome.jp/tools/motif/)* with an E-value cut off score of 1.0 in Pfam database [27].

RESULTS

Bioinformatics Study and Genome Comparison

Bioinformation of 11 selected *M. bovis* strains was collected in this study *(Table 1)*. With the updated annotation information from NCBI, 750 open reading frames (ORFs) were identified in *M. bovis* Ningxia-1 genome (total 920,475 bp, max 9,981 bp and min 114 bp) which occupied 89.05% of the whole genome, with an average length of 1,227 bp and a mean GC content of 29.74%. 629 out of 750

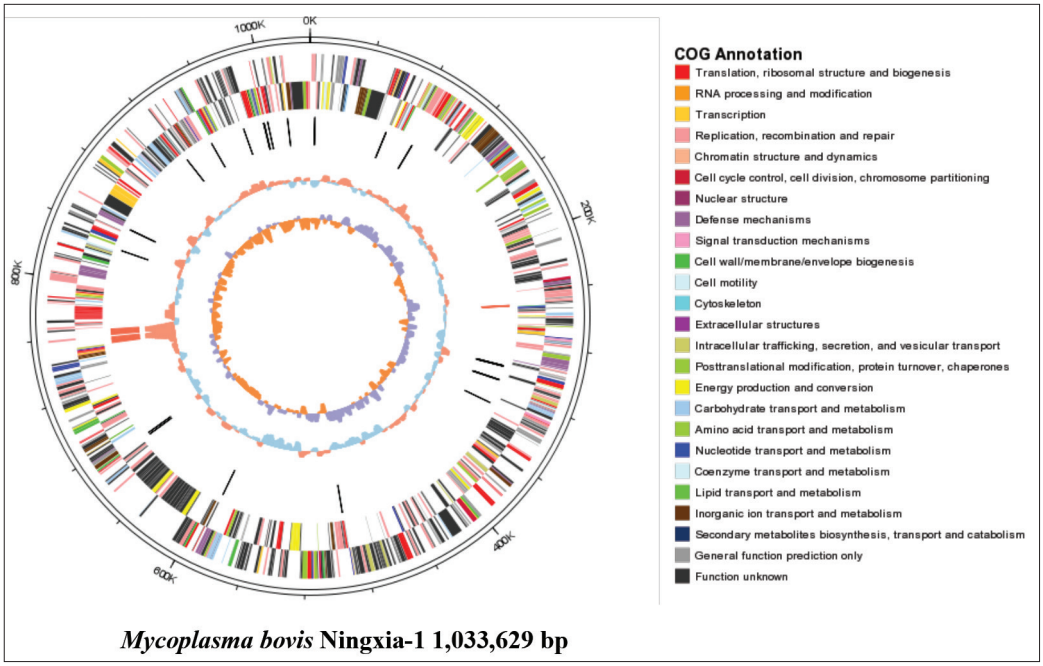


Fig 2. Circular Diagram of the *M. bovis* Ningxia-1 Genome Structure. The genome of *M. bovis* Ningxia-1 contains a single circular chromosome of 1,033,629 bp, with a GC content of 29.3%. A total of 887 genes and 750 open reading frames (ORFs) were identified. Ninety-four pseudogenes were predicted by GeneMarkS+. The genome encodes 6 rRNA and 34 tRNA genes, which representing all 20 amino acids. The outer black circle shows the whole genome length. Moving inside, the first and second circles show predicted coding sequences (CDSs) on the plus and minus strands respectively. Gold for translation, ribosomal structure and biogenesis; orange for RNA processing and modification; light orange for transcription; dark orange for DNA replication, recombination and repair; antique white for cell division and chromosome partitioning; pink for defense mechanisms; red for signal transduction mechanisms; peach for cell envelope biogenesis and outer membrane; deep pink for intracellular trafficking, secretion and vesicular transport; pale green for post-translational modification, protein turnover and chaperones; royal blue for energy production and conversion; blue for carbohydrate transport and metabolism; dodger blue for amino acid transport and metabolism; sky blue for nucleotide transport and metabolism; light blue for coenzyme metabolism; cyan for lipid metabolism; medium purple for inorganic ion transport and metabolism; aquamarine for secondary metabolites biosynthesis, transport and catabolism; and gray for general function prediction only; black for unknown function). The third circle shows tRNA (black) and rRNA (red). The fourth circle shows the content of G+C (red: above mean, blue: below mean). The fifth circle shows G+C skew (purple: above mean, orange: below 0)

(83.9%) of these genes could be classified into Clusters of Orthologous Groups (COG) families which have 19 functional categories *(Fig. 2)*. The genome encodes 6 rRNAs and 34 tRNAs genes which represent all 20 amino acids. The comparison of 11 selected *M. bovis* strain genomes is shown in *Table 2*.

Identification of 24 Housekeeping Genes through Literature Annotation and Evolutionary Position

Through manual literature annotation, we identified reports on 24 housekeeping genes that have been determined and applied in experiments. For each gene in the list, the following information was recorded: (1) paper PMID; (2) gene symbol; (3) nucleotide sequence; (4) protein sequence; and (5) identifier. The 24 housekeeping genes identified in this study were all found in STRING database (Supplement Table S1).

We identified 24 genes in 11 orthologous strains, *adk*, *atpA*, *dnaA*, *dnaK*, *dnaN*, *efp*, *fusA*, *gltX*, *gmk*, *gpsA*, *gyrB*, *lepA*, *metG*, *polC*, *pta_1*, *recA*, *rpoB*, *rpoD*, *tdk*, *tkt*, *tpiA*, *tuf* and *uvrC*. Phylogenetic trees were created for each of the 24 genes between 11 *M. bovis* stains and *M. agalactiae* strain to examine evolutionary position, which indicated a close relationship between *M. bovis* Ningxia-1 and NM2012 *(Fig. 3)*.

BLAST Homology Between M. bovis Ningxia-1and M. agalactiae

Each of the 24 identified genes was BLASTed against *M. bovis* Ningxia-1 strains along with the *M. agalactiae* whole genome. The homology between *fusA*, *dnaK*, *tkt* and *tuf* genes is over 90%, which indicates a high similarity between *M. bovis* Ningxia-1 and *M. agalactiae*, whereas the similarity of *adk*, *gpsA*, *polC* and *uvrC* is below 83%, which indicates the possibility for those genes to be used as a clinical detection marker to differentiate *M. bovis* and *M. agalactiae (Table 3)*. *adh-1*, *atpA*, *dnaA*, *dnaN*, *efp*, *gltX*, *gmk*, *gyrB*, *lepA*, *metG*, *pta_1*, *recA*, *rpoB*, *rpoD*, *tdk* and *tpiA* of *M. bovis*, fall between 83-90% homology range also making them potential candidates for use in diagnosis.

STRING Protein-Protein Analysis of M. bovis Ningxia-1

In order to predict interaction networks between the 24 housekeeping genes in *M. bovis* Ningxia-1 strain each

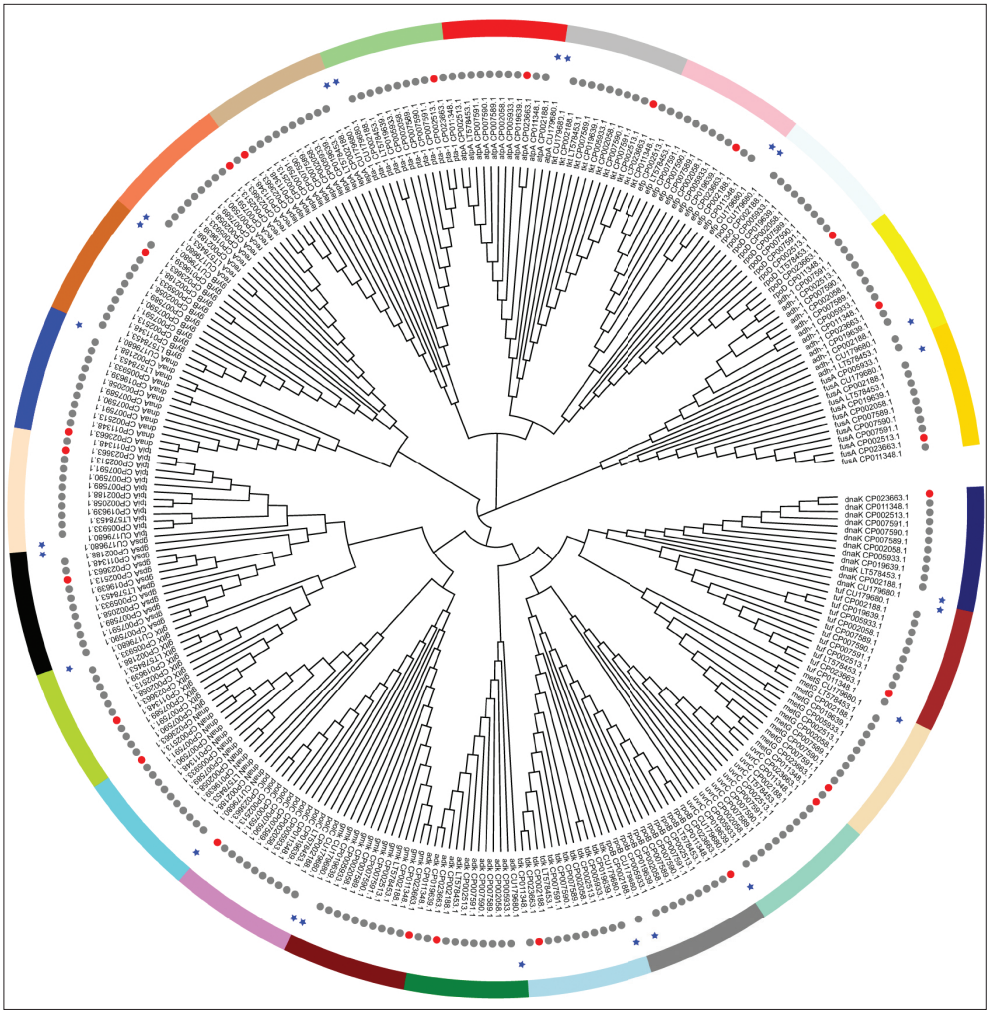
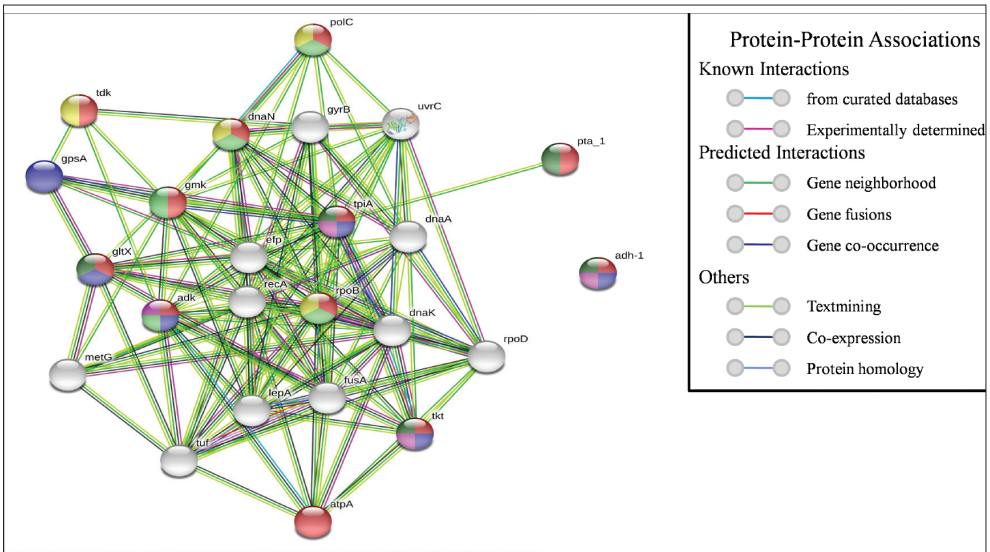


Fig 3. Phylogenetic position of 24 selected housekeeping genes from 11 selected *M. bovis* strains and 1 exogenous reference strain *M. agalactiae*. *M. bovis* Ningxia-1 has a close relationship with NM2012, PG45, JF4278 and Hubei-1. The red dot represented *M. bovis* Ningxia-1strain, the grey indicated other homology *M. bovis*. *M. agalactiae* was marked by blue stars. The different strip color represented 24 genes, yellow is for *adh-1*, green is for *adk*, red is for *atpA*, blue is for *dnaA*, navy is for *dnaK*, cyan is for *dnaN*, pink is for *efp*, gold is *fusA*, greenyellow is for *gltX*, maroon is for gmk, black is for *gpsA*, chocolate is for *gyrB*, tan is for *lepA*, wheat is for *metG*, violet is for *polC*, lightgreen is for *pta_1*, coral is fro *recA*, gray is for *rpoB*, azure is for *rpoD*, lightblue is for *tdk*, silver is for *tkt*, bisque is for *tpiA*, brown is for *tuf*, aquamarine is for *uvrC*

was analyzed using STRING. These networks include 130 interactions, with expected number of edges to be 90 based on the evidence view. Such enrichment indicates that the proteins are at least partially biologically connected, as a group. 12 proteins including *adh-1, adk, atpA, dnaN, gltX, gmk, polC, pta_1, rpoB, tdk, tkt* and *tpiA*, are involved in metabolic pathways. 6 proteins, *adh-1, adk, gltX, gpsA, tkt* and *tpiA*, are involved in biosynthesis of secondary metabolites. 4 proteins including *adh-1, adk, tkt and tpiA* may be involved in the biosynthesis of antibiotics. 5 proteins, *adh-1, gltX, pta_1, tkt* and *tpiA*, are involved in microbial metabolism in diverse environments while 2 sets of genes are involved in nucleotide metabolism process: 5 proteins, *adk, dnaN, gmk, polC* and *rpoB* perform in purine metabolism and 4 proteins, *dnaN, polC, rpoB* and *tdk* have function in pyrimidine metabolism *(Fig. 4)*. Under the confidence view with active interaction sources of experiments and databases, only four proteins *adk, atpA, rpoB* and *rpoD* are present in the results. *Adk* has 4 functions in KEGG pathways (pathway ID: mbv01100, mbv01110, mbv00230, mbv01130), including metabolic pathways, biosynthesis of secondary metabolites, purine metabolism, biosynthesis of antibiotics. *AtpA* is only involved in metabolic pathways. *Adk* and *atpA* participated in curated pathways in relevant datasets in *M. bovis*: adenosine ribonucleotides de novo biosynthesis, bioCyc/ecoCyc Pathways *(www.biocyc.org)*. RpoB has 3 functions in KEGG pathways (pathway ID: mbv01100, mbv00230 and mbv00240), including metabolic pathways, purine metabolism and pyrimidine metabolism. No experimental/biochemical data was shown, but putative homologs were found interacting in other species (score 0.990). RpoB and rpoD show relevant information with *Escherichia coli K12* MG1655.

Table 3. *Homology of 24 housekeeping genes between M. bovis and M. agalactiae*

Gene Name	Search Sequence	Query Sequence	Identity	Size	Different	E Value	Total Score
adh-1	CP023663.1:430952-432001	CU179680.1	84	1050	168	0	1137
adk	CP023663.1:c751508-750858	CU179680.1	82.642	651	113	0	665
atpA	CP023663.1:c528856-527270	CU179680.1	85.381	1587	232	0	1817
dnaA	CP023663.1:1000-2400	CU179680.1	85.796	1401	199	0	1630
dnaN	CP023663.1:2535-3644	CU179680.1	84.054	1110	177	0	1205
efp	CP023663.1:592716-593279	CU179680.1	88.652	564	64	0	729
fusA	CP023663.1:c840005-837912	CU179680.1	92.311	2094	161	0	3051
gltX	CP023663.1:820782-822173	CU179680.1	86.135	1392	193	0	1641
gmk	CP023663.1:272425-273012	CU179680.1	84.014	588	94	0	637
gpsA	CP023663.1:c69556-68558	CU179680.1	80.08	999	199	0	905
gyrB	CP023663.1:1005729-1007696	CU179680.1	85.018	1969	293	0	2214
dnaK	CP023663.1:185269-187065	CU179680.1	90.095	1797	178	0	2439
lepA	CP023663.1:698096-699889	CU179680.1	87.786	1793	219	0	2247
metG	CP023663.1:c268839-267289	CU179680.1	84.521	1544	239	0	1708
polC	CP023663.1:83679-88055	CU179680.1	81.536	4387	791	0	4223
pta_1	CP023663.1:173625-174581	CU179680.1	88.506	957	110	0	1231
recA	CP023663.1:783965-784948	CU179680.1	86.154	975	135	0	1150
rpoB	CP023663.1:c873691-870056	CU179680.1	89.741	3636	373	0	4876
rpoD	CP023663.1:c365396-363867	CU179680.1	88.374	1531	176	0	1952
tdk	CP023663.1:c957009-956437	CU179680.1	83.877	552	89	4.94E-171	595
tkt	CP023663.1:256009-257955	CU179680.1	93.066	1947	135	0	2903
tpiA	CP023663.1:c743511-742729	CU179680.1	87.101	783	100	0	954
tuf	CP023663.1:c578649-577459	CU179680.1	96.725	1191	39	0	1973
uvrC	CP023663.1:725657-727372	CU179680.1	82.761	1717	294	0	1755



KEGG Pathways

#Pathway ID	Pathway description	Observed gene count	False discovery rate	Colour	Matching proteins in your network (labels)
mbv01100	Metabolic pathways	12	0.00068		adh-1,adk,atpA,dnaN,gltX,gmk,polC,pta_1,rpoB,tdk,tkt,tpiA
mbv01110	Biosynthesis of secondary metab	6	0.0086		adh-1,adk,gltX,gpsA,tkt,tpiA
mbv00230	Purine metabolism	5	0.0086		adk,dnaN,gmk,polC,rpoB
mbv00240	Pyrimidine metabolism	4	0.0394		dnaN,polC,rpoB,tdk
mbv01130	Biosynthesis of antibiotics	4	0.042		adh-1,adk,tkt,tpiA
mbv01120	Microbial metabolism in diverse	5	0.042		adh-1,gltX,pta_1,tkt,tpiA

Fig 4. Analysis of 24 housekeeping genes is to define the essential genes in *M. bovis* strain Ningxia-1 compared to PG45 isolate, to prevent cross reaction during identification. 24 selected protein sequences were put into the STRING database to generate potential protein-protein interactions, using the type strain of *M. bovis* PG45 as the database default reference. Different colored lines show different types of interactions. Different colored lines show different types of interactions e.g., textmining (emerald green), experiments (violet), databases (sapphire), neighborhood (green), gene fusion (red), co-occurrence (purple), co-expression (black) and etc. In evidence view, all possible interactions are shown

Table 4. *Most frequent Motifs among M. bovis Ningxia-1 strain housekeeping genes*

Motif	Genes	Description
MMR_HSR1	*dnaA, fusA, lepA, tuf*	PF01926, 50S ribosome-binding GTPase
GTP_EFTU_D2	*fusA, lepA, tuf*	PF03144, Elongation factor Tu domain 2
GTP_EFTU	*fusA, lepA, tuf*	PF00009, Elongation factor Tu GTP binding domain
ABC_tran	*adk, dnaA, recA*	PF00005, ABC transporter
AAA_14	*atpA, dnaA, recA*	PF13173, AAA domain
AAA	*adk, dnaA, recA*	PF00004, ATPase family associated with various cellular activities (AAA)

Transmembrane Analysis of Housekeeping Genes of M. bovis Ningxia-1

To determine transmembrane probability for each housekeeping gene, their protein sequences were pasted into TMHMM. TMHMM v 2.0 is the most popular software in the field [28], with the ability to distinguish cytoplasmic membrane and outer domains in a hidden Markov model [29]. Our results demonstrate that *metG* is very likely to be a transmembrane protein and the total probability that the N-term is on the cytoplasmic side of the membrane. The rest of 23 housekeeping genes are outer membrane-associated protein in *M. bovis*.

Motif Analysis of Housekeeping Genes

Table 4 lists the top common motifs in the housekeeping gene proteins of *M. bovis* Ningxia-1 strain. The 50S ribosome-binding GTPase family motif PF01926 is present in 4 housekeeping gene proteins *(dnaA, fusA, lepA* and *tuf)* and is the most common motif among all housekeeping genes. Concurrent with the high frequency motif of elongation factor Tu domain 2 and elongation factor Tu GTP binding domain are also found in *fusA, lepA* and *tuf*. ABC transporter and ATPase family associated with various cellular activities (AAA) motifs are found in *adk, dnaA* and *recA*. Additionally, AAA domain is found in proteins encoded by *atpA, dnaA* and *recA* of *M. bovis* Ningxia-1. Finally, we analyzed a less frequent but equally interesting set of genes linked by two genes for example the 3-hydroxyacyl-CoA dehydrogenase (NAD binding domain), AAA ATPase domain and UDP-glucose/GDP-mannose dehydrogenase family (NAD binding domain), are three motifs found in many proteins of *M bovis (adh-1, gpsA, dnaA* and *recA)*. tRNA synthetases class I (C) catalytic domain is only shown to have less impact on *metG*.

DISCUSSION

Mycoplasma. bovis is one of actively evolving mycoplasmas [30]. *M. bovis* PG45 strain was identified in the USA six decades ago, whereas the Hubei-1 and HB0801 strains were detected in China during 2008. An inversion has been found in the two strains isolated in China. It is assumed that a long interval and the geographical variation may be a cause for this inversion [30]. In contrast with the *M. bovis* genome, housekeeping genes are stable and perform basic fundamental functions and evolve more slowly in terms of both coding and core promoter sequences [17].

Many efforts have been made to identify and characterize the surface proteins in *M. bovis* [31]. However, little is known regarding hierarchy among experimentally proven housekeeping genes, or the distribution and the number of motifs within these genes in *M. bovis*. To date, there is no consensus among the current databases for *M. bovis* and from our understanding many of these predicted surface membrane proteins are yet to be definitively identified and the functions of most of them have not been determined [32]. Despite this though, several membrane proteins and lipoproteins are used in diagnostic assays for detection of antibodies specific for *M. bovis* [32].

16S rRNA gene is a small subunit within prokaryotic ribosomes, commonly used for bacterial identification [33] [Yang, 2016 #1128][Yang, 2016 #1128]. *M. bovis* infections are typically diagnosed by isolation and identification of causative agent and confirmed by the presence of the 16S rRNA gene using PCR [5]. Despite the 16s rRNA gene-based PCR possessing great specificity, cross-amplification of *M. agalactiae* can still occur [34]. A highly stable gene, uvrC, encodes an enzyme essential for replication and involved in DNA repair, known as deoxyribodipyrimidine photolyase [35]. The *uvrC* gene has no cross amplification with non-*M. bovis* species including *M. agalactiae,* proving it is a well conserved and much more specific target gene than 16S rRNA gene [36,37]. However, point mutations in the *M. bovis uvrC* gene have been identified in recent studies, making false negative PCR results identify *M. bovis* strains [38]. Meantime, many novel genes such as *fusA* (encodes for elongation factor G and require in the translation process of mRNA into proteins) genes were also developed to use as detection markers [39]. *RpoB* (encoding the β-subunit of RNA polymerase), is another core gene candidate for phylogenetic analyses and identification of bacteria, especially of closely related isolates [40]. But from our BLAST results, *fusA* has 92.3% and *rpoB* has 89.7% identity with *M. agalactiae*, may not contribute as a diagnosis marker to differentiate *M. bovis* and *M. agalactiae*. Our findings on the 24 housekeeping genes show *adk*, *gpsA*, *polC* also could be used as potential

detection genes to differentiate between *M. bovis* species and *M. agalactiae*.

STRING is more liberal with assigning interactions, as it uses data from homologous protein interactions in different schemas. These interactions suggest that these proteins might function together, and thus, based on bioinformatics methods, we can have a further analysis for these genes. A homology comparison feature is incorporated into STRING, which makes it easier to determine the function of un-identified *M. bovis* genes. In addition to these features which we used in this study, STRING also has other useful features, including a feature that allows for homology comparisons in a phylogenetic context, and protein family analysis. TMHMM utilizes a hidden Markov model to determine transmembrane domains on proteins, with the ability to distinguish cytoplasmic and outer domains and is currently one of the most accurate membrane protein topology prediction methods [29]. Motif analysis could provide us the path to determining the motifs present in the housekeeping genes.

Identification of the essential specific genes and their motifs in the host could benefit us to develop drugs and vaccines against *M. bovis* infection [41,42]. Therefore, future work should focus on identifying these housekeeping genes, especially for the *adk*, *gpsA* and *polC*, which have great potential to benefit the insight of *M. bovis*, and be used as an improved detection tool for clinical diagnosis, *metG* gene could be an important virulence gene based on in silico prediction as a trans-membrane gene and *adk*, *dnaA*, *fusA*, *lepA* and *recA* would extend the treatment method.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Author Contributions

P.S. and Y.F. designed and performed experiments, analyzed results, and wrote the manuscript. Q.W. and M.Y. provided advice. S.H. and X.S. supervised the study and wrote the manuscript.

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Research Article

Sustainable Increase of Cow Milk Productivity Using Components of Siberian Forest and Alpha-Amylase Enzyme

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Abstract

At the places of harvesting coniferous trees and industrial nuts processing remains, a devastating amount of wastes litters large areas of land, poisons the air and the hydro-environment by the combustion products and toxic compounds released during decomposition. Therefore, the search for solution for the effective use of waste is an actual task. The aim of this research is to investigate the influence of fodder additive made of forest waste resources and the enzymes on milk productivity of cows. The experiment has been held on black-and-white breed in Krasnoyarsk region. The experimental group received, in addition to the main diet, some fodder additives consisted of coniferous flower, crushed pine nut shells, arabinogalactane and amilosubtiline HCl enzyme. The analysis of coniferous flower demonstrated that it contains more copper by 41.6%, zinc-55.6%, magnesium-183.3%, chromium-66.7% than in pine nut shell thus, on the contrary, the content of lignin, cellulose and moisture, was more in the shell of pine nuts-24.2%, 9.7% and 5.0%, respectively. Under the effect of the test additives in cows of the experimental group milk yield was higher than the control group, 9.9% ($P<0.05$), fat mass fraction - 0.3% ($P<0.05$), the amount of milk basis of fat content 19.0%, in the blood was higher total protein content 2.7%, glucose - 4.0; albumin - 3.0; - creatinine 6.0; triglycerides by 11.1%; calcium 1.5%; phosphorous - 10.2; iron - on 3.6%, magnesium - 2.6%. Thus, the tested additives had a positive synergistic effect on the body of cows, which affected the increase in the quantity and quality of milk.

Keywords: *Coniferous flower, Pine nut shell, Arabinogalactane, Amilosubtiline HCl, Milk efficiency, Cow*

Sibirya Orman Ürünleri ve Alfa-Amilaz Enzimi Bileşenlerini Kullanarak İneklerde Süt Veriminin Sürdürülebilir Artışı

Öz

İğne yapraklı ağaçlar ve endüstriyel fıstık işleme alanlarında geniş arazileri kirleten, yanma ürünleri ile hava ve hidro-ortamı zehirleyen, ayrışma sırasında açığa çıkan toksik bileşiklerden oluşan yıkıcı miktarda atık kalır. Bu nedenle, bu atığın etkili kullanımı için çözüm aramak önemli bir sorumluluktur. Araştırmanın amacı, orman atık ürünleri ve enzimlerden yapılan yem katkılarının ineklerin süt verimi üzerindeki etkisidir. Deney Krasnoyarsk bölgesindeki siyah-beyaz ırkta yapıldı. Deney grubuna ana diyete ek olarak iğne yapraklı çiçek, ezilmiş çam fıstığı kabuğu, arabinogalaktan ve amilosubtilin HC1 enziminden oluşan bazı yem katkı maddeleri verildi. İğne yapraklı çiçeğin analizi, çam fıstığı kabuğundan %41.6, çinko - %55.6, magnezyum - %183.3, krom - %66.7 oranında daha fazla bakır içerdiğini gösterdi, aksine lignin, selüloz ve nem içeriği, çam fıstığı kabuğunda sırasıyla - %24.2, %9.7 ve %5.0 daha fazlaydı. Katkı maddelerinin kullanıldığı deney grubundaki ineklerde süt verimi kontrol grubundan daha yüksekti, %9.9 ($P<0.05$), yağ kütlesi fraksiyonu - %0.3 ($P<0.05$), yağ içeriğinin süt bazlı miktarı %19.0, kandaki toplam protein içeriği %2.7, glikoz - 4.0; albümin - 3.0; - kreatinin 6.0; trigliseritler %11.1; kalsiyum %1.5; fosfor - 10.2; demir - %3.6, magnezyum -%2.6'ydı. Bu nedenle, söz konusu katkı maddelerinin, inekler üzerinde, süt miktarı ve kalitesinde artışla sonuçlanan pozitif bir sinerjistik etkisi olduğu belirlendi.

Anahtar sözcükler: *İğne yapraklı çiçek, Çam fıstığı kabuğu, Arabinogalaktan, Amilosubtiline HCl, Süt verimi, İnek*

INTRODUCTION

Highly productive cows have the most intensive metabolism associated with the formation of a large number of nutrients released from milk. They have increased activity of the cardiovascular, respiratory, endocrine and other systems of the body. The quantity and quality of milk to a large extent depend on the amount of food eaten, its chemical

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composition and the balance of the diet on nutrients. Sometimes it is almost impossible to ensure high productivity of animals only at the expense of feed of own production. Therefore, it is advisable to use feed additives containing various nutrients and biologically active substances that can enrich the diet [1].

Krasnoyarsk territory has one of the largest reserves of forest resources among the regions of Russia [2]. Coniferous wood species - Siberian larch *(Lárix sibírica* L.) and common pine *(Pinus sylvestris)* prevail in terms of logging and wood harvesting on the territory of Siberia [3]. Forest enterprises of the timber industry mainly use stem wood, which is 60-65% of the total biomass of the tree.

Coniferous browses and butt of the tree are the wastes left on the cutting areas without any usage [4]. It negatively affects the environment in places of the felling, and prevents the growth of forests [5], by forming wastes of wood processing that are harmful to the environment associated with the contamination of large tracts of land, poisoning the air and hydrosphere combustion products landfills, toxic compounds released during decomposition, etc [3]. At the same time, from each hectare of logging, coniferous browses can be collected in an amount that is enough to produce 10-12 tons of coniferous flour [6] and a large number of unclaimed butts of the tree that accumulates into waste [7].

Coniferous needle contains carotene, sugar, glucose, fructose, pectin, tannins, cobalt, copper, manganese, zinc, iron, and vitamins B, C, E, and K [6]. Such a rich complex mineral and vitamin nutrition of coniferous flour allows it to show antimicrobial, immunostimulating, antioxidant, anti-inflammatory, hematopoietic action and can be successfully used to stimulate the growth and development of farm animals, increase productivity and improve its quality [8].

The butt of the tree is the most enriched with the polysaccharide arabinogalactane, the content of which reaches 15-20 % by weight [7].

Arabinogalactane is a complex natural water-soluble polysaccharide extracted from larch wood [1,9]. It is larch that makes it possible to obtain arabinogalactane with the most useful properties [10].

The use of this polysaccharide as a feed additive can increase the productivity of farm animals. When exposed to the intestinal micro flora, polysaccharides break down into short-chain fatty acids, which in ruminants are considered the main source of glucose in the blood, which can significantly reduce the energy deficit in the body [11].

A special place in the structure of the natural resources of Siberia is occupied by cedar forests, the main asset of which was the pine nut [12].

Harvesting pine nuts for food purpose has a long traditional trade of the population in Siberia. For one ton of harvested pine nuts is more than two tons of waste remain [13], a significant amount of which falls on its shell (51-59% of the weight of the nut) [14]. The presence of mineral substances in the nut shell and a high content of carbohydrates characterize it as a source of carbohydrate-mineral complex [15].

In the Krasnoyarsk Territory annual 40.07 million tons of green conifers waste is formed. According to the Federal State Statistics Service management for the Krasnoyarsk Territory, in 2019 the number of cattle in farms of all categories amounted to 368.4 thousand heads, including 141.2 thousand heads of cows.

Considering of the foregoing premises, the use of forest industry waste, arabinogalactane and enzymes in the feeding of dairy cows is relevant.

The aim of the research is to study the effect of a fodder additive from forest resources and an enzyme on the dairy productivity of cows.

MATERIAL and METHODS

Ethics Statement

This study was carried out in accordance with the «Rules for the use of experimental animals "(Appendix to the order of the Ministry of Health of the USSR dated 08/12/1977, No. 755)). Federal state budget scientific institution "Federal research centre "of the Krasnoyarsk scientific center of the Siberian branch of the Russian Academy of Sciences. (protocol approval number: 7 - 09.12.2019)

The experiment has been held on lactating cows of black-and-white breed in "Breeding Farm Taezhny" Ltd, Sukhobuzimsky area of Krasnoyarsk region.

To conduct the experiment due to analogue concept there were formed two groups of black-and-white breed lactating cows of the second calving 15 animals in each group *(Table 1)*.

The experimental cows were of average fatness (BCS = 3.5), average live weight was 600-620 kg, average yield of milk - 24 kg/day, DIM - from 11 to 100 d in milk. The animals were kept in a typical four-row barn with a year-round

Table 1. *Experience scheme*

Group	Amount of Cattle	Duration (days)	Feed Condition
Control	15	100	Basic Diet (BD)
Experimental	15	100	BD + coniferous flower (50 g/h) + pine nut shell (50 g/h) + arabinogalactane (5 g/h) + amilosubtiline HCl enzyme (5 g/h)

stall system. Each cow was tied (THS), had a separate stall, individual feeder, and drinker.

The animals of the control group received a basic diet contains of haylage of perennial grasses-20 kg, straw-6 kg, barley-2 kg, wheat-2.1 kg, oats-2.5 kg, rapeseed cake-0.8 kg, sunflower cake-1 kg, grain molasses-1.8 kg.

In addition to the main diet, the cows of the experimental group were fed a mixture consisting of coniferous flour, pine nut shells, amilosubtiline hc (hydrochloride) enzyme and arabinogalactane once a day morning. The main diet was given to each cow individually. Experimental additives were daily mixed with concentrated feed (barley, wheat, oats) and given to cows of the experimental group in dry. The TMR system was not used on this farm. Composition and food value of the main diet presented in the *Table 2*.

Observations of the animals in the experimental groups showed that they willingly and without residue consumed concentrated feed containing test additives.

The main diet contained: energetic feed unit (EFU) - 20.3; exchange energy - 203.8 MJ; digestible protein - 2163.3 g; dry matter - 20.46 kg; crude fat - 776.1 g; crude fiber - 4060.7 g; sugar - 1998 g; methionine - 95.5 g; tryptophan - 28.7 g; calcium - 125 g; phosphorus - 76.9 g; magnesium - 42.2 g; carotene - 986.7 mg.

In 1 kg of dry matter of the diet contained 0.99 EFU, carotene - 48.23 mg, digestible protein per 1 EFU - 106.6 g, the sugar-to-protein ratio was 0.92: 1, the calcium-to-phosphorus ratio was 1.4: 1. The dry matter of the diet contained: crude fiber - 19.81%, raw fat - 3.8%, sugar - 9.8%.

Coniferous flour was made from pine branches *(Pinus sylvestris)*, which were subjected to extraction with an alcohol-toluene mixture and hot water on an extraction plant, then dried and ground to a state of crumbly flour. Pine nut shells were crushed to a particle size of no more than 4 mm. Preparation of coniferous flour and crushed pine nut shells, as well as their analysis were carried out in a physical and chemical laboratory of wood plants biology in Forest Institute. named after V. N. Sukachev SD RAS.

Table 2. *Composition and food value of the main diet*

Indicator	Feed								Total
	Haylage Perennial Herbs	Straw	Barley	Wheat	Oats	Sunflower Cake	Oilseed Rape	Molasses Rom Rye Grain	
Amount of feed, kg/day	20.0	6.0	2.0	2.1	2.5	1.0	1.8	1.8	37.38
EFU	7.8	2.0	2.4	2.2	2.3	1.0	0.9	1.7	20.3
Exchange energy, mJ	78.0	20.4	23.6	22.5	23.0	10.4	9.0	16.9	203.8
Dry matter, kg	9.0	2.7	1.78	1.79	2.13	0.9	0.72	1.44	20.46
Digestible protein, g	744.0	60.0	222.0	298.2	197.5	324.0	209.6	108.0	2163.3
Crude Fat, g	408.0	60.0	30.0	31.5	100.0	77.0	69.6	0	776.1
Crude fiber, g	2538.0	942.0	60.0	58.8	242.5	129.0	90.4	0	4060.7
Starch, g	94.0	90.0	1120.0	1029.0	800.0	25.0	0	0	3158
Sugar, g	696.0	138.0	30.0	31.5	62.5	62.6	0.0	977.4	1998
Lysine, g	52.0	8.4	10.4	8.2	9.0	13.4	12.6	0	114
Methionine, g	46.0	8.4	4.4	8.6	8.0	15.8	4.3	0	95.5
Tryptophan, g	8.0	1.8	3.6	2.9	2.8	5.2	4.4	0	28.7
Calcium g	74.0	29.4	0.8	1.5	3.8	5.9	3.8	5.8	125
Phosphorus, g	26.0	7.8	6.0	9.0	8.5	12.9	6.3	0.4	76.9
Magnesium, g	16.0	7.8	4.6	2.3	3.0	4.8	3.5	0.2	42.2
Potassium, g	136.0	70.2	10.2	9.7	13.5	9.5	8.9	59.2	317.2
Sulfur, g	16.0	5.4	0	0.8	3.5	5.5	3.6	2.5	37.3
Copper, mg	74.0	30.6	16.6	4.8	12.3	17.2	5.8	8.3	169.6
Zinc, mg	210.0	87.0	62.4	84.0	56.3	40.0	38.8	37.4	615.9
Manganese, mg	386.0	222.6	85.0	86.3	141.3	37.9	35.4	44.3	1038.8
Cobalt, mg	0.6	1.2	0.2	0.0	0.2	0.2	0.2	1.1	3.7
Iodine, mg	1.6	0.6	0	0.2	0.3	0.4	0.3	1.3	4.7
Carotene, mg	810.0	150.0	0	21.4	3.3	2.0	0	0	986.7
Vitamin D ME	2700.0	1080.0	0	0	0	5.0	2.4	0	3787.4
Vitamin E, mg	760.0	210.0	0.0	27.9	32.3	11.0	9.6	5.4	1056.2

Arabinogalactane is produced in JSC "Ametis" (Blagoveshchensk, Amur region). To activate cicatricial digestion, coniferous flour, pine nut shells and arabinogalactane were mixed with amilosubtiline hc (hydrochloride) enzyme produced in PE "Sibbiopharm" (Berdsk, Novosibirsk region).

The animals participating in the experiment were kept in identical conditions in separate stalls using a tie. Milking cows on the farm is carried out in the milk delivery line (a milk line).

Quality determining of composition components (volatile compounds) of samples of coniferous flour and pine nut shells was performed in the Krasnoyarsk Regional Centre of common use FITC KNC SB RAS on the chromato-mass spectrometer "Agilent 5975C-7890A" (USA) using a vapor-phase sampler HeadSpace Sampler G 1888.

Milk productivity was established by conducting daily control milking. The study of chemical indicators of milk and biochemical indicators of blood were carried out at the beginning and at the end of the experiment.

The mass fraction of fat and protein in milk were determined on the milk analyzer "Lactoscan FARM Eco" (Bulgaria).

The suitability of milk for cheese production was determined by the rennet clotting to GOST 32901-2014. The method is based on the ability of raw milk to coagulate under the influence of rennet and microorganisms of raw milk. According to the results of formed clots visual assessment, raw milk was assigned to one of three classes: class I - a clot with a smooth surface, elastic to the touch, without eyes in a longitudinal section, floats in a transparent, non-stretching milk serum; II class - a clot soft to the touch with single eyes (1-10), torn, but not swollen; Grade III - a clot with numerous eyes, spongy, soft to the touch, swollen, floated up.

Blood for biochemical analysis was taken from the tail vein after morning feeding using special needles of vacuum tubes with a coagulation activator (PUTH, PRC). Blood serum of cows was examined by photometric method on the biochemical and immunoassay blood analyzer "Chem Well 2910 c" (USA).

Setting up and conducting the experiment was carried out by the method of Ovsyannikov [16].

Analysis was done by the method of Plokhinsky [17] using the computer program "Analysis Package for biometric processing of zootechnical data" [18]. Statistical differences between the two groups were calculated with Student's t-test. The level of significance was $P<0.05$, $P<0.01$, $P<0.001$.

RESULTS

Pine nut shell had a rich brown color with a pronounced smell of pine nuts, coniferous flour-yellow had a green color with a smell of pine needles.

In comparison with pine nut shell the coniferous flour had more extractive substances extracted by alcohol-toluene mixture by 23.4%, hot water-by 16.5%, while the amount of lignin, cellulose and moisture, on the contrary, was greater in the shell of pine nuts-by 24.2%; 9.7 and 5.0%, respectively *(Table 3)*.

Mineral substances *(Fig. 1)* in coniferous flour is more than in pine nut shell: level of copper by 41.6%, zinc - 55.6%, magnesium - 183.3%, chromium - 66.7%

Table 4 presents milk efficiency of cows for the initial 100 days of lactation.

The result of feeding tested additives confirms that the animals of the experimental group has higher milk yield than the control group by 9.9% ($P<0.05$), the mass fraction of fat - by 0.3% ($P<0.05$), the amount of milk of basic fat content - by 19.0%.

According to the physical and chemical characteristics

Table 3. *Physical and chemical characteristics of coniferous flour and pine nut shell*

Characteristic	Coniferous Flour	Pine Nut Shell
Chemical composition		
Monosaccharide, mg/g totally dry weight	0.30	traces
Oligosaccharide, mg/g totally dry weight	8.65	2.37
Starch, mg/g totally dry weight	1.15	0.79
Physical-chemical components		
Moisture %	6.7	11.7
Extractive substances extracted from the sample: hot water, % alcohol-toluene mixture, %	 29.66 20.57	 4.06 6.26
Polyphenols, g/L	0.19	0.14
Cellulose, %	27.15	36.82
Lignin, %	20.49	44.64

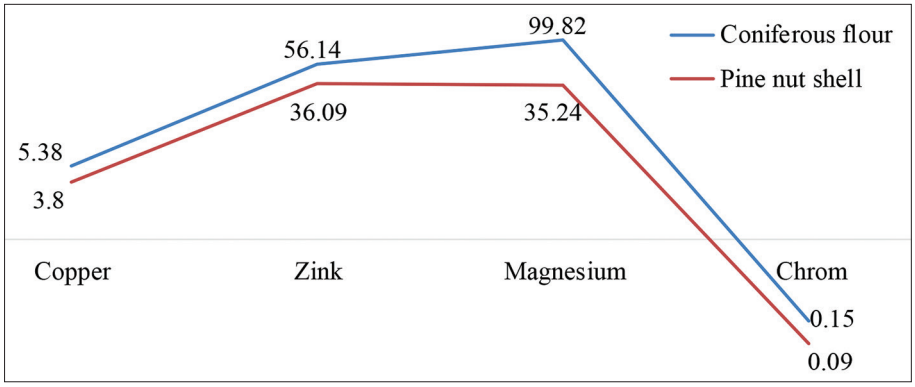

Fig 1. Mineral substances in coniferous flour and pine nut shell

Table 4. *Milk yield efficiency (M±m, n=15)*

Indicator	Group	
	Control	Experimental
Milk yield for 100 days of lactation, kg	2085.36±44.95	2292.65±71.06*
Average daily yield, kg	20.85±0.45	22.93±0.71*
Mass fraction of milk fat, %	3.49±0.07	3.78±0.10*
Mass fraction of protein, %	3.19±0.09	3.19±0.14
FCM (fat corrected milk, 3.4%), kg	2139.51	2546.58
ECM, kg	1936.52	2206.19
**P<0.05*		

Table 5. *Shows the physical and chemical characteristics of milk at the end of the experiment (M±m)*

Indicator	Group	
	Control	Experimental
Mass fraction of milk fat, %	3.49±0.23	3.90±0.32
Mass fraction of protein, %	3.18±0.52	3.42±0.45
Lactose, %	5.15±0.66	5.54±0.56
Mass fraction of MSNF (milk solids non-fat), %	8.35±0.09	8.26±0.16
Salts, %	0.66±0.34	0.28±0.30
Dry component, %	11.10±0.22	11.91±0.27
Water, %	1.14±1.27	1.02±0.03
Density, kg/м^3	1028.27±0.45	1027.73±0.87
Active acidity (pH)	6.48±0.01	6.41±0.03
Urea, mg/100ml	4.40±1.36	8.24±1.90
Temperature of freeze, °C	0.549±4.87	0.544±1.92

studied at the end of the experiment *(Table 5)*, has been found that the milk of the experimental group cows has higher characteristics in comparison with the control group, such as the mass fraction of fat - by 0.41%, protein - by 0.24%, lactose - 0.39%, dry component - 0.81%, urea - 87.3%.

The results of milk testing for cheese production presented in *Table 6*.

This experience clearly demonstrates that in the experimental group, under the influence of the tested additives, the proportion of cows whose milk corresponded to the first class of cheese suitability increased and amounted to 100%.

Curd of milk in the experimental group better than in the control group and had a smoother surface, clear shape, density and elasticity *(Fig. 2)*.

Highly productive cows are sensitive to various changes in the feed diet, which is invariably reflected in animal blood composition [19]. As a result of biochemical studies of the blood of cows at the end of the experiment *(Table 7)*, it was found that all the studied blood serum parameters did not exceed the maximum permissible values, but there

Table 6. *Classification of milk for cheese suitability, %*

Group	At the Beginning			At the End		
	Class Cheese Suitability					
	I	II	III	I	II	III
Control	20	40	40	20	60	20
Experimental	0	80	20	100	0	0

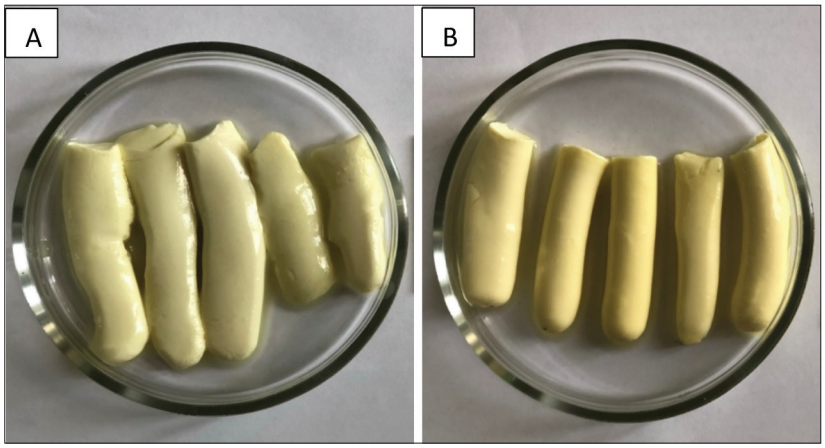

Fig 2. Curd of milk of tested cows. **A-** Control group, **B-** Experimental group

Table 7. *The results of biochemical blood parameters of cows (M±m)*

Parameter	Group	
	Control	Experimental
Protein, g/L	83.64±2.27	85.90±3.96
Glucose, mmol/L	3.52±0.23	3.66±0.28
Albumin mmol/L	31.72±1.39	32.66±0.79
Creatinine, mmol/L	94.18±8.58	99.84±3.41
Triglyserids, mmol/L	0.09±0.02	0.10±0.02
Cholesterol, mmol/L	5.22±0.45	5.20±0.68
Amylase, Un/L	13.00±3.48	12.40±2.28
Chlorides, mmol/L	98.00±2.76	93.80±1.39
Calcium, mmol/L	2.67±0.12	2.71±0.15
Phosphorus, mmol/L	2.26±0.19	2.49±0.24
Iron, mmol/L	28.18±0.91	29.18±0.81
Magnezium, mmol/L	1.16±0.02	1.19±0.01

were minor deviations between the groups. In animals of the control group, the level of cholesterol, amylase and chlorides was higher than in the experimental group by 0.4%; 4.8 and 4.5%, respectively. In the experimental group, compared with the control group, the total protein content was higher by 2.7%, glucose-by 4.0%; albumin-by 3.0%; creatinine-by 6.0%; triglycerides-by 11.1%; calcium-by 1.5%; phosphorus-by 10.2%; iron-3.6%, magnesium-2.6%.

The calculation of economic efficiency showed, the cost of used feed additive consisting of coniferous flour, pine nutshell, arabinogalactan, and the enzyme preparation Amilosubtilin G3x for the full period of the experiment per cows amounted to 845 rubles. The use of these additives in cow feeding allowed to increase milk productivity by 9.9%, reduce the single cost of milk by 7.9%, and increase the level of profitability by 8.4%.

DISCUSSION

As far as we know, this is the first research of studying the complex influence of coniferous flour, pine nut shells, arabinogalactane and amilosubtiline hc (hydrochloride) enzyme for cow milk efficiency

Previously, we conducted research on feeding cows

coniferous flour and pine nut shells [20,21]. The obtained data had a positive effect and allowed to obtain a patent for the invention [22]. Studying the effectiveness of each component separately on the body of cows allow us to see the validity of their presence in a diet.

Coniferous flour was fed to animals by researchers [23], but according to their results it had an adverse effect on the animal's body and caused an abortion. However, a team of other scientists [24] on the contrary claims that the ading of coniferous flour into a cow's diet did not cause abortions.

More over it contributed to the growth of antioxidant protection [25]. Due to the high antibacterial characteristics of coniferous needles, there is a decrease in the content of somatic cells in the milk of cows [26].

Researchers who used pine nut shells in the diet of laboratory animals claim to increase endurance of cows [27]. Despite the fact that there are quite few studies about feeding it, we have found evidence of a positive effect of the tested component on the calves [28]. The authors claim that adding of pine nut shell into the diet of calves normalizes digestion and metabolism, as well as increases the activity of the body's defenses. This is also confirmed by our research [29], the results show the positive obtained effect.

Using amilosubtiline HCl enzyme, which main part is α-amylase enzyme, is quite effective in the diet of lactating cows. It is evidenced by the results of researchers [30,31], claiming a tendency of increasing milk productivity while using it. The researchers [32] suggest that this enzyme can increas the productivity of animals by increasing the digestion of starch, while the amount of starch does not necessarily increase. However, other researchers have not found this effect determind milk productivity [33], noticing that the absorption of nutrients has increased.

According to researchers [34,35] the use of arabinogalatane as a feed additive has a positive effect on the level of natural resistance of the body and on the formation of intestinal microflora. In a more detailed study of the use of arabinogalactane, the authors [36] noticed its structure of macromolecules, which consists of 95-99% of arabinogalactane and 1-5% of proteins and has prebiotic characteristics. On its base some prebiotic additives for supporting the growth of bifidobacteria and lactobacilli have been developed. Tests on calves conducted by the authors [37] defined that adding of arabinogalactane in the diet helps to improve the efficiency of nutrition and has a positive effect on the hematological status, increasing weight gain. However, the main mechanism of positive influence on newborn calves is its ability to provide a high level of humoral and cellular immunity. In the diet of animals, the presence of arabinogalactane contributed to the strengthening of adaptive and protective functions of the body, and it is also proved that such diets have anti-atherogenic properties [38].

The study on feeding cows coniferous flour (50 g/head/day), pine nut shells (50 g/head/day), arabinogalactane (5 g/head/day) and amilosubtiline HCl enzyme (5 g/head/day) indicate that their complex use had a synergistic effect, resulting in increased milk yield by 9.9% ($P<0.05$), the mass fraction of fat - by 0.3% ($P<0.05$), the amount of basic fat content of milk - by 19.0%, as well as improved technological properties of milk and blood biochemical parameters.

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Statement of Author Contributions

EI - conducting an experiment, studying the components of a feed additive, examining the blood of animals, processing the data obtained, writing the manuscript of the article; OI - determination of the research topic, control of the analysis of results, statistical processing of research results, interpretation of research results; VT - development and conduct of an experiment, the study of blood and milk of animals, statistical processing of research results; LE - analysis of animal milk.

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Research Article

Development of a SYBR Green Real-Time PCR Assay with Melting Curve Analysis for Simultaneous Detection of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*

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Abstract

In the present study, a duplex SYBR Green real-time PCR assay was developed in order to indentify *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* infection in one reaction, through a melting curve analysis. This method utilized two pairs of specific primers that allowed the amplification of highly conserved regions of *A. pleuropneumoniae* Apx IV and *H. parasuis* omp P2 gene. Reconstitution experiments were conducted by using PMD - 19T plasmid in order to determine the sensitivity of the assay. The results showed that the Tm values of the melting curves of *A. pleuropneumoniae* and *H. parasuis* were 83.36±0.09ºC and 76.48±0.17ºC, respectively that could accurately distinguish these two pathogens. And no cross reaction were observed between other respiratory pathogens, which suggested a high specificity of two primers. The detection sensitivity of the assay was 127 and 96 copies/µL which was higher than that of the ordinary PCR detection methods. This rapid technique may present a simple, useful option for simultaneous detection of *A. pleuropneumoniae* and *H. parasuis*, which would be feasible and attractive for clinical samples diagnosis and epidemiological investigations.

Keywords: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, SYBR Green I, Real-time PCR, Melting curve*

Actinobacillus pleuropneumoniae ve *Haemophilus parasuis*'in Eşzamanlı Saptanması Amacıyla Erime Eğrisi Analizi İle SYBR Green Real-Time PCR Testinin Geliştirilmesi

Öz

Bu çalışmada, bir erime eğrisi analizi yoluyla tek bir reaksiyonda *Actinobacillus pleuropneumoniae* ve *Haemophilus parasuis* enfeksiyonunu tanımlamak için bir dubleks SYBR Green gerçek zamanlı PCR analizi geliştirildi. Bu yöntemde, *A. pleuropneumoniae* Apx IV ve *H. parasuis* omp P2 geninin yüksek oranda korunmuş bölgelerinin amplifikasyonuna izin veren iki çift spesifik primer kullanıldı. Analizin duyarlılığını belirlemek için PMD - 19T plazmid kullanılarak sulandırma deneyleri gerçekleştirildi. Sonuçlar, *A. pleuropneumoniae* ve *H. parasuis*'in erime eğrilerinin Tm değerlerinin sırasıyla bu iki patojeni doğru şekilde ayırt edebilen 83.36±0.09ºC ve 76.48±0.17ºC olduğunu gösterdi. Diğer solunum sistemi patojenleri ile aralarında çapraz reaksiyon gözlenmedi, bu da iki primerin yüksek özgüllüğünü ortaya koydu. Testin saptama hassasiyeti, sıradan PCR yöntemlerinden daha yüksek olan 127 ve 96 kopya/µL idi. Bu hızlı teknik, *A. pleuropneumoniae* ve *H. parasuis*'in eşzamanlı tespiti için basit ve kullanışlı bir seçenek sunabilir;i söz konusu seçenek klinik örneklerin teşhisi ve epidemiyolojik araştırmalar için uygulanabilir ve cazip olacaktır.

Anahtar sözcükler: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, SYBR Green I, Real-Time PCR, Erime eğrisi*

INTRODUCTION

Actinobaccillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia, which is characterized by serious bleeding and fibrinous pleuropneumonia [1]. The disease is susceptible to various pigs and accompanies

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with high morbidity and mortality. To date, 15 serovars of *A. pleuropneumoniae* have been described. Some *Streptococcus pleuropneumoniae* strains can also cause recessive infections in local herds. Most of them are secondary infections in swine respiratory disease syndrome, which increase the herd mortality under the combined action of other viruses or bacteria [2]. Rapid and accurate determination of the involved pathogen is important for both limiting the severity of an outbreak and tracing the source of the infectious agent. Molecular methods based on genetic differences of *A. pleuropneumoniae*, such as PCR, MPCR have been developed to detect *A. pleuropneumoniae* [3,4]. Furthermore, two real-time PCR assays were constructed to detect *A. pleuropneumoniae* serovars 1-9-11 and serovar 2, which are useful for the serotyping of *A. pleuropneumoniae* in diagnostic laboratories to control porcine pleuropneumonia [5].

Haemophilus parasuis, the causative agent of Glasser's disease, which is characterized by fibrinous polyserositis and arthritis. In addition to Glasser's disease, *H. parasuis* produces other clinical outcomes, such as pneumonia, and colonizes the upper respiratory tract of healthy animals in herds in which acute infections occur only sporadically [6]. In recent years, the immunosuppression caused by various reasons has worsened the occurrence of *H. parasuis* infection, which also easly develops secondary to *A. pleuropneumoniae* and aggravates the disease [7]. The isolation of *H. parasuis* is not as simple as general techniques due to the fragility and fastidious growth requirements of the bacterium. Coupled with classical diagnostic methods, various molecular methods have been developed recently to characterize *H. parasuis* strains [8,9]. PCR was shown to be more sensitive than cultivation.

A. pleuropneumoniae and *H. parasuis* are important pathogens in the respiratory tract of pigs, and the clinical symptoms they cause are difficult to distinguish [10]. Multiple infections also cause difficulties in traditional bacteriological diagnosis. It is necessary to establish a rapid diagnosis and identification method to distinguish the two bacteria. "Repeats in toxin" (RTX) and some toxins (ApxI, ApxII, ApxIII and ApxIV) are involved in the virulence of many organism including *A. pleuropneumoniae*. Unlike other three toxins, ApxIV toxin has been demonstrated to be a specific marker of *A. pleuropneumoniae* infection [11]. Outer membrane protein (OMP) P2 is the most abundant protein in the outer membrane and is a crucial virulent factor of *H. parasuis*. It has been found that OMP P2 belonged to 2 genotypes and is highly conserved in all of the published sequences of *H. parasuis* [12]. A real-time PCR assay based on OMP P2 gene was developed, and performed similar to previously described other diagnostic tool [8].

In this study, according to the each conservative area of *A. pleuropneumoniae* Apx IV and *H. parasuis* omp P2 gene, two pairs of primers were designed, and after the specificity and sensitivity test, a duplex fluorescent quantitative PCR detection method was developed to identify *A. pleuropneumoniae* and *H. parasuis*.

MATERIAL and METHODS

Strains

A. pleuropneumoniae (ATCC27088), *Escherichia coli (ATCC25922)*, *Salmonellae choleraesuis (ATCC10708D-5)*, *Pasteurella multocida (ATCC 31610)*, *Streptococcus aureus (ATCC25923)* and *H. parasuis (ATCC 19417)* were maintained in our laboratory (Microbiology Laboratory of College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology). *E. coli*, *S. choleraesuis* and *Streptococcus aureus* were cultivated in nutrition agar medium and broth medium at 37°C for 12 h. *A. pleuropneumoniae, P. multocida* and *H. parasuis* were cultivated in tryptic soy broth (TSB, Oxoid) accompany with 1% nicotinamide adenine dinucleotide (NAD, Sigma-Aldrich)and 10% fetal bovine serum (FBS, Sigma-Aldrich) at 37°C for 18 h.

Primers

According to the conserved sequences of *A. pleuropneumoniae* Apx IV gene and *H. parasuis* ompP2 gene published in GenBank database, two pairs of primers were designed for quantitative fluorescence detection through the Primer 5.0 software. The primers were synthesized by Sangon (Shanghai, China). The sequence of two pairs of primers is shown in *Table 1*.

DNA Extraction

Bacterial DNA extraction from organ and cell supernatants was performed using a MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's instructions. The purity and concentration of the extracted DNA samples were determined by NanoDrop 2000 spectro-photometer (NanoDrop 2000, Thermo Scientific, Unite state) and stored at -80°C.

Table 1. *Primers for real-time PCR assay used in this study*

Genes	Primer	Sequence (5'-3')	Tm/°C	Length
Apx IV	Forward	GCAACAACGTCGCACAAT	55.4	90bp
	Reverse	GAAGCAGCCAACTCCTCAG	55.3	
Omp P2	Forward	AATCGGTGATAGCATTGGTC	54.8	218bp
	Reverse	ACACTTCTCCCTTATTATTACGC	55.1	

Standard Recombinant Plasmid Construction

The PCR were performed in a thermocycler with 100ng DNA as template in a 20 µL reaction volume containing 1 µM of each primer and 1 U Taq DNA polymerase (Promega, Unite State). The amplification reactions were performed according to the following protocol: 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 53°C for 45 s, 72°C for 2 min, and a final elongation step of 10 min at 72°C. The equal volume of deionized water was added (replace the template DNA) as a negative template control (NTC) reactions. PCR products were examined by electrophoresis on a 1.5% (w/v) agarose gel and visualized after ethidium bromide (EB). PCR products of the expected length were purified with a Gel Extraction kit (OMEGA, USA), then cloned into the PMD19-T vector (TaKaRa, Japan) according to the manufacturer's instructions and sequenced at BGI (Beijing, China). After the plasmid identification was correct, the plasmid was extracted using a Plasmid mini Kit (OMEGA, USA) followed the manufacturer's procedure. The copy number of extracted plasmid was calculated according to the formula: copies/µL = (6.02x10^{23}) x (plasmid concentration ng/µLx10^{-9})/(DNA lengthx660). The plasmid concentration was measured by NanoDrop 2000 spectrophotometer.

Simplex and Duplex SYBR Green Real-Time PCR Assays

Each simplex PCR reaction was carried out in a total reaction volume of 10 µL containing 5 µL 2×SYBR green PCR master mix (ABI, US), 1 µM of each primer and 100ng of DNA template and then adjusted to final volume with double-distilled water. PCR conditions for simplex PCR were: an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C 15 s and 72°C for 20 s. The amplified DNA was detected by fluorescence quantification of the double-stranded DNA binding dye SYBR Green with melting curve analysis. The melting curves were acquired at 72°C for 15 s in 1 cycle and then an increase in temperature to 95°C at 0.1°C/s.

The duplex PCR reaction was carried out according to previously described [13]. Briefly, in a total volume of 20 µL in one tube containing 10 µL 2×SYBR green PCR master mix (ABI, US), 1 µM forward and reverse primer of *A. pleuropneumoniae* and 1 µM forward and reverse primer of *H. parasuis*, and 100ng DNA template of *A. pleuropneumoniae*, 100ng DNA template of *H. parasuis* and the final volume was adjusted with deionized water. NTC reactions were carried out simultaneously. The melting curve and PCR condition were similar to simplex PCR. Data were analyzed using ABI QuantStudio 5 Gene software version.

Standard Curve and Limit of Detection

The simplex real-time PCR standard curve was generated for both *A. pleuropneumoniae* and *H. parasuis* by serial 10-fold dilutions of the two recombinant plasmids with a known copy number (from 1x10^{10} to 1x10^{1} copies/µL). These dilutions were tested in triplicate and used as quantification standards to construct the standard curve by plotting the plasmid copy number against the corresponding threshold cycle values (Ct value). The PCR efficiency (E) was calculated using the standard curve slope according to the following formula: E=($10^{(-1/slope)}$ -1). The correlation coefficient (R^2) was also calculated. A melting curve analysis of the obtained amplification products was carried out.

Sensitivity, Specificity and Repeatability Analysis

The analytical sensitivity of real-time PCR is the lowest amount of sample (the highest dilution of plasmid) which can be detected. Genomic DNA of *E. coli*, *S. choleraesuis*, *P. multocida*, *Str. aureus*, *A. pleuropneumoniae* and *H. parasuis* stored in our laboratory was extracted and employed as a template for specific detection of duplex SYBR green real-time PCR assay. 100ng DNA of each of the above-mentioned bacteria was mixed and used as template to evaluate the specificity of the dual fluorescence quantitative PCR method. All amplification reactions were performed in duplicate in three independent experiments.

The repeatability of the melting temperature analysis was examined by comparing the results obtained from the replicates of three dilutions (10^4, 10^5, 10^6 copies/µL) of both *A. pleuropneumoniae* and *H. parasuis* recombinant plasmids during a single PCR reaction and comparing the mean values obtained from the same plasmids on three different days.

Clinical Sample Testing

Clinical samples of lung materials from 37 pigs with respiratory system infection symptoms were collected, detected and compared respectively by conventional PCR and fluorescent-based quantitative PCR.

RESULTS

Simplex Real-Time PCR Assay

We cloned the expected PCR amplicons (90bp and 218bp) into PMD-19T to constructed *A. pleuropneumoniae* and *H. parasuis* recombinant plasmid respectively. The linearity and efficiency of the SYBR Green real-time PCR were determined by generating a standard curve for each of the two pathogens *(A. pleuropneumoniae* and *H. parasuis)* in which serial 10-fold dilutions of recombinant plasmids were tested. The standard curve was generated by plotting the real-time PCR threshold cycle numbers (Ct) of each dilution against the known copy numbers of recombinant plasmid. The resulting slope showed a linear relationship over 10 orders of magnitude ranging from 1x10^{1} to 1x10^{10} copies/µL for both *A. pleuropneumoniae* and *H. parasuis*

standard plasmids. From the *Fig. 1-A,* the slope was -3.4951 with a coefficient of determination (R2) >0.99 and reaction efficiency (E) of 0.932 for *A. pleuropneumoniae* and -3.7076 with a R2 >0.99 and an E of 0.861 for *H. parasuis*. The LOD determined on the *H. parasuis* standard curves was found to be 127 and 96 copies/µL respectively, thus showing a high sensitivity of the assay.

The optimal concentration of primers for each of these two pathogens was found to be 1 µM. Amplicon melting curve showing a single peak for each set of primers suggests that non-specific amplification products or primer dimmers (results not shown) for each of the two pathogens confirms the specificity of the reaction. The melting temperature analysis showed a variation of Tm values between samples of 83.36°C±0.09 and 76.48°C±0.17 for *A. pleuropneumoniae* and *H. parasuis*, respectively *(Fig. 1-B)*.

These two plasmids with three serial dilutions were tested at different times and under the same conditions. Each sample was repeated three times, and the coefficient of variation of the Ct value was calculated. The results in *Table 2* showed that the intra-assay coefficient of variation and inter-assay coefficient of variation of the three samples were between 0.16% and 0.32%; both less than 10%, indicating that the method was reproducible.

Duplex Real-Time PCR Assays

Duplex assay was developed using two recombinant plasmids in one tube. Optimal primer concentrations for the duplex assays were 1 µM of each primer. The melting curve showed two melting peaks corresponding to the expected Tm values of the simplex PCR assay of *A. pleuropneumoniae* and *H. parasuis (Fig. 2-A)*. The primers did not cross react with other pathogens *(Fig. 2-B)*. This duplex real-time assay enabled the simultaneous detection of *A. pleuropneumoniae* and *H. parasuis* in co-infected samples in a single tube PCR assays.

Validation of Real-Time PCR Assays Using Field Samples

To evaluate the practical utility and accuracy of the duplex real-time PCR assay, a total of 37 susceptive lung samples were tested for the presence of these two bacteria species. Based on the melting curve, 20 samples displayed a peak with a Tm value of 83.4, and 9 samples displayed a peak with a Tm value of 76.6, and 6 samples displayed two peaks with Tm value of 83.4 and 76.6. In contrast, only 4 samples were positive for two bacteria when diagnosed using conventional PCR detection method *(Table 3)*. These results indicate that the duplex real-time PCR assay is more sensitive than bacterial isolate for the detection of co-infection from field samples.

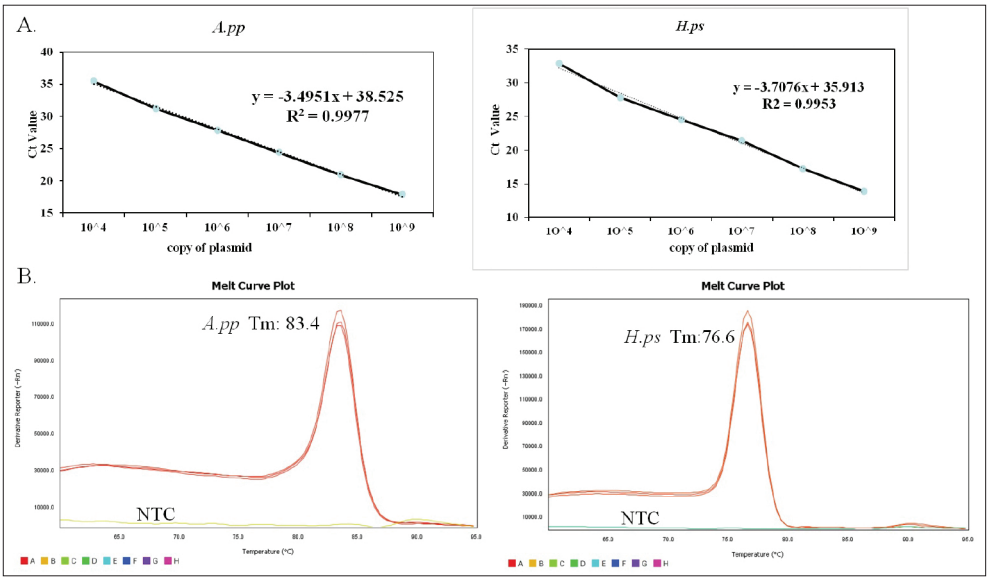

Fig 1. Simplex real time PCR assay of *A. pleuropneumoniae* and *H. parasuis.* **(A)** Standard curves for *A. pleuropneumoniae* and *H. parasuis* using recombinant plasmid template DNA, **(B)** Melting curve analysis showing the melting temperature peaks (Tm) of *A. pleuropneumoniae* and *H. parasuis* and negative template controls (NTC); N=3

Table 2. *Intra- and inter-assay variations of Tm values (°C) obtained from real-time PCR with melting curve analysis*

Pathogens	Intra-assay Variation			Inter-assay Variation		
	Mean	SD	CV (%)	Mean	SD	CV (%)
A. pleuropneumoniae	76.63	0.16	0.22	76.64	0.24	0.32
H. parasuis	83.48	0.12	0.16	83.47	0.17	0.23

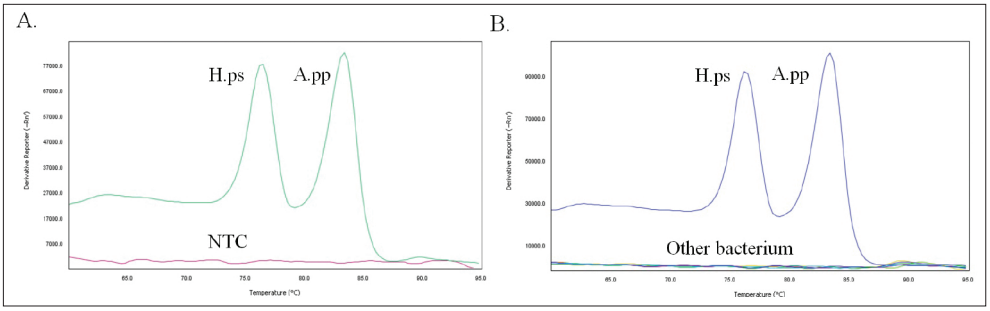

Fig 2. Duplex real time PCR assay. **(A)** Separation of amplicon melting peaks in SYBR® Green duplex real-time quantitative reverse transcription PCR assays. Targets are indicated above each peak along with non-target negative controls (NTC), **(B)** Specificity of duplex real time PCR assay with other respiratory bacterium; N=3

Table 3. *A. pleuropneumoniae and H. parasuis duplex real-time PCR methods were compared with conventional PCR methods*

Method	***A. pleuropneumoniae***	***H. parasuis***	***A. pleuropneumoniae* + *H. parasuis***
Conventional PCR method	19	9	4
Real-time PCR	20	9	6

DISCUSSION

Although several end points PCR have been described previously for the detection and differentiation of *A. pleuropneumoniae* and *H. parasuis*, they usually involve two separate reactions for each bacterium or further procedures (such as DNA sequencing) are needed for species-specific identification [14]. These additional procedures cause consumption of additional time and cost more, thus delay the results. In our research, simplex and duplex SYBR Green based real-time were optimized for the detection of two pathogens. In contrast, TaqMan-based assays are more highly target gene specific than dyes of SYBR® Green in real time PCR assay. However, considering the cost, the assays developed in this study provide a rapid and cost-effective alternative to TaqMan qPCR with similar efficiency and accuracy, particularly useful when large numbers of samples are to be analyzed such as epidemiological investigation.

SYBR® Green in combination with melting curve analysis (MCA) has been successfully used to detect the co-infection of Canine adenovirus type 1 (CAdV-1) and canine adenovirus type 2 (CAdV-2) [15]. In our study, the two primers employed in this research yielded a Tm difference of nearly 7°C (83.4°C, 76.6°C) between the reference strains of *A. pleuropneumoniae* and *H. parasuis* which were most suitable for identifying the two pathogens. This obvious difference in Tm values for *A. pleuropneumoniae* and *H. parasuis* makes it easy to simultaneous identification of these two pathogens in one reaction. This reliable and rapid technique may represent a simple, useful and economic option for simultaneous detection of *A. pleuropneumoniae* and *H. parasuis*.

Compared to Real-time PCR, the conventional PCR is still widely used since it is cheap and easy to operate. The conventional duplex PCR with agarose gels can easily identify two different amplicons in one reaction [16]. Furthermore, these specific two primers involved in our real-time PCR assay can also be used in conventional duplex PCR assay with detection of amplicons by agarose gels for detection and differentiation of *A. pleuropneumoniae* and *H. parasuis* for resource-limited mycology laboratories that do not have a real-time PCR machine. These data suggest that these two primers can be widely used.

In conclusion, the real-time PCR method based on melting point analysis with SYBR Green dye described here is a simple and rapid molecular tool for accurate detection and differentiation of strains of *A. pleuropneumoniae* and *H. parasuis*. In case of multiple infections, the assays will provide valuable information on epidemiological investigation and diagnosis.

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Author Contributions

Conceived and designed the experiments: Y. DONG, L. WANG, S. ZHANG. Performed the experiments: B. Hu, Y. XU, Z. WANG, Q. REN, J. XU. Analyzed the data: B. HU, Y. XU. Wrote the paper: B. HU, S. ZHANG.

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Research Article

The Role of Viral and Parasitic Pathogens Affected By Colony Losses in Turkish Apiaries

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Abstract

Parasites and viruses are considered major agents of bee colony loss. The aim of this study is to detect the presence of seven viruses and three parasites - namely, Israeli acute bee paralysis virus (IAPV), deformed wing virus (DWV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV), chronic bee paralysis virus (CBPV), *Nosema, Varroa* and *Tropilaelaps* spp. - in Turkish apiaries affected by colony loss. For this purpose, nine apiaries were examined in 2016, 38 in 2017 and 29 in 2018. The results show that DWV was the most prevalent virus in Turkish apiaries, identified in 44.7% of the samples. The second-most widespread virus was ABPV, in 35.5% of the samples, while the prevalence of BQCV, SBV, CBPV and IAPV was 28.9%, 22.3%, 18.4% and 6.5%, respectively. However, KBV was not identified in any of the apiaries. With regard to microsporidian and parasitological investigations, *Nosema* was found in 10, *Varroa* was detected in 21 and both *Varroa* and *Nosema* were simultaneously identified in seven apiaries. *Tropilaelaps* spp. was not detected. In some examples, two, three, four and even five viral agents were detected simultaneously. Mixed viral infections were common in some colonies, which had between two and five different viruses. In conclusion, viruses and mixed infections in particular play a major role in declining colonies.

Keywords: *Apis mellifera, Bee viruses, Colony losses, Nosemosis, Turkey, Varroasis*

Koloni Kaybından Etkilenen Türk Arılıklarında Viral ve Paraziter Patojenlerin Rolü

Öz

Parazitler ve viruslar koloni kayıplarına sebep olan önemli etkenlerdir. Bu çalışmada Türkiye arılıklarından koloni kaybı şikayeti ile gelen örnekler yedi viral etken; İsrail akut arı felci virusu (IAPV), deforme kanat virusu (DWV), sacbrood virusu (SBV), akut arı felci virusu (ABPV), siyah kraliçe hücre virusu (BQCV), kaşmir arı virusu (KBV), kronik arı felci virusu (CBPV) ve paraziter etkenler olan *Nosema, Varroa* ve *Tropilaelaps* spp. yönünden incelenmiştir. Bu amaçla 2016 yılından dokuz, 2017 yılından 38 ve 2018 yılından ise 29 örnek teste tabi tutulmuştur. Koloni kaybı yaşanan arılıklarda en fazla tespit edilen virus %44.7 ile DWV olmuştur. İkinci en yaygın virus ise %35.5' le ABPV'dir. BQCV, SBV, CBPV ve IAPV prevalansı ise sırasıyla %28.9, %22.3, %18.4 ve %6.5 olarak bulunmuştur. KBV ise örneklerin hiçbirinde tespit edilmemiştir. Parazitolojik incelemeler sonucunda *Nosema* 10 arılıkta belirlenirken, *Varroa* 21 arılıkta tespit edilmiştir. Yedi arılıkta ise hem *Nosema* hem de *Varroa* belirlenmiştir. *Tropilaelaps* spp. ise örneklerin hiçbirinde bulunmamıştır. Bazı örneklerde ikili, üçlü, dörtlü ve hatta beşli viral etkenler aynı anda tespit edilmiştir. Sonuç olarak, koloni kayıplarına yol açabilecek birçok faktör vardır, özellikle virüsleın ve miks enfeksiyonların azalan kolonilerde önemli bir rol oynadığı düşünülmektedir.

Anahtar sözcükler: *Apis mellifera, Arı virusları, Koloni kaybı, Nosemosis, Turkey, Varroasis*

INTRODUCTION

Honeybees *(Apis mellifera L.)* are economically and biologically indispensable due to their balancing and unique pollinator roles in the ecosystem [1]. The total global value of crops pollinated for food production by honeybees was estimated at nearly €153 billion [2]. Unfortunately, significant colony losses have recently been

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observed in honeybee populations [3]. There was a loss of approximately 10 million bees in the United States in the winter of 2006-2007, when an agent causing the sudden disappearance of all adult bees in a hive led to colony collapse disorder (CCD) [4]. It is believed that the reduction in honeybee populations is caused by parasitic diseases, viruses, microsporidians, genetic factors, severe seasonal weather conditions, malnutrition and exposure to pesticides [4-9].

Honeybee colony losses are a multifactorial phenomenon that include biotic and abiotic causes [10]. Pathogens such as viruses and parasites are biotic factors. Most important honey bee pathogens known to affect colony health have also been defined by ANSES (French Agency for Food, Environmental and Occupational Health And Safety). These are; a predatory beetle *(Aethina tumida)*, two ectoparasitic mites *(Varroa destructor* and *Tropilaelaps* spp.), two fungi *(Nosema apis, N. cerenae)*, two bacteria which cause American foulbrood and European foulbrood *(Paenibacillus larvae and Melissococcus plutonius)* and three viruses (CBV, DWV, and ABPV).

Varroosis is a disease of honeybees caused by mites in the genus Varroa. It is the most important honeybee disease to affect honeybees and cause destructive losses in colonies. The Varroa mite can be observed on both adult bees and brood. This mite acts as a vector for viruses (particularly DWV) [11,12]. *Tropilaelaps* spp. causes similar damage as *Varroa* infestations; it is a parasite of the brood of large honeybees, such as *Apis dorsata, A. laboriosa, and A. breviligula*. As in *Varroa*, it has been reported that it causes colony losses especially when heavy infestation in *A. mellifera* [13]. In addition, this mite can also act as a vector for viruses of the honeybees [14,15]. *Nosema* is an extremely important and common honeybee disease caused by the microsporidia *Nosema apis* and *N. ceranae*. It is transmitted through ingestion of spores by adult bees [16]. The epidemiology of BQCV is generally associated with *Nosema* infection [17,18].

More than 20 distinct viruses have been identified that affect the genus *Apis* [19]; this number changes continuously with the identification of new viruses, such as apis rhabdovirus-1, apis rhabdovirus-2 [20] and varroa orthomyxo-virus-1 [21]. Bee viruses in general belong to one of two families: Iflaviridae or Dicistroviridae. They are non-enveloped, single-stranded and positive-sense RNA viruses [1]. With the exception of the *Apis mellifera* filamentous virus and the *Apis* iridescent virus, which have DNA genomes, all of these viruses are single-stranded RNA viruses. Apart from chronic bee paralysis virus, which has an isometric shape, most of these viruses cannot be distinguished using particle morphology [22]. Israeli acute bee paralysis virus (IAPV), deformed wing virus (DWV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV) and chronic bee paralysis virus (CBPV) are the most common viruses that cause infections in honeybee colonies [1,23].

Turkey has significant beekeeping potential, with 7.4 million colonies, and ranks 3rd in the world after China and India, which is proof of the importance of Turkey in the global trade of beekeeping [24]. Scientists and beekeepers are intensely preoccupied honey bee colony health problems for more than a decade in Turkey [5,25]. So it is important to investigate colony losses. The objective of this study was to determine the presence of pathogens affecting colony losses in Turkish apiaries.

MATERIAL and METHODS

Sample Preparation

Alive or dead honey bee samples were sent by complaints with colony losses. A total of 2280 honey bee samples from 76 different apiaries *(Fig. 1)* that occurred in seven provinces (Adana, Aydın, Bursa, Izmir, Kütahya, Muğla and Manisa) of Turkey between 2016 and 2018 were tested. For this purpose, nine apiaries were examined in 2016, 38 in 2017 and 29 in 2018.

Fig 1. The geographical location of the apiaries affected by colony losses in seven provinces

Spore and Parasite Detection by Microscopic and Macroscopic Investigation

To determine infestations of both *Varroa* and *Tropilaelaps spp*. mites, a pool was created from approximately 100-300 honey bees after adding 70% ethyl alcohol to remove mites from the honeybee. Then the jar was sealed and shaken for several min. Floating mites were then observed on a surface with 70% ethyl alcohol.

To identify *Nosema spp*. spores, at least 60 adult bee abdomens were masserated in 20-30 mL sterile distilled water, then the suspension was centrifuged for 10 min at 3500 rpm and after that 2-3 drops of the suspension were placed on a slide under a coverslip and examined with the light microscope at 400x magnification (Olympus BX53).

RNA Extraction

Thirty adult bees were homogenized with 9 mL Eagle Minimum Essential Medium (EMEM) (Sigma, U.K.) followed by centrifugation for 30 min at 3500 rpm and 4°C. The supernatant was stored at -80°C until analyzed. For RNA extraction 200 µL of supernatant was used. Total RNA extraction was carried out using High Pure Viral RNA Kit (Roche, Germany) following the manufacturer's instructions.

Table 1. *Primers used in this study*

Agent	Primer Pairs	Amplicon Length	Ref.
IAPV-F IAPV-R	*CGAACTTGGTGACTTGAAG* *GCATCAGTCGTCTTCCAGG*	110 bp	[4]
DWV-F DWV-R	*TGGTCAATTACAAGCTACTTGG* *TAGTTGGACCAGTAGCACTCAT*	269 bp	[26]
SBV-F SBV-R	*CGTAATTGCGGAGTGGAAAGATT* *AGATTCCTTCGAGGGTACCTCATC*	342 bp	[26]
AIV-F ABPV-R	*GGTGCCCTATTTAGGGTGAGGA* *ACTACAGAAGGCAATGTCCAAGA*	460 bp	[26]
BQCV-F BQCV-R	*CTTTATCGAGGAGGAGTTCGAGT* *GCAATAGATAAAGTGAGCCCTCC*	536 bp	[26]
AIV-F AIV-R	*GGTGCCCTATTTAGGGTGAGGA* *TGCACGGGAAGTATAAATAATTCT*	641 bp	[27]
CBPV-F CBPV-R	*AACCTGCCTCAACACAGGCAAC* *ACATCTCTTCTTCGGTGTCAGCC*	774 bp	[26]

PCR Amplification

The target fragments of IAPV, DWV, SBV, ABPV, BQCV, KBV, CBPV were amplified using specified primers for each virus as detailed in *Table 1*. The total volume of each PCR reaction was 50 µL, consisting of 30 µL of water, 10 µL of reaction buffer (One-step transcriptor, Roche), 2 µL of forward primer (10 p/mol), 2 µL of reverse primer (10 p/mol), 1 µL of Taq polymerase (Roche) and 5 µL of RNA. Cycling was a multi-step process that included one cycle at 50°C for 30 min for reverse transcription followed by initial denaturation at 95°C for 7 min, then denaturation at 95°C for 20 sec, followed by annealing at 56°C for 20 sec, extension at 72°C for 30 sec for 45 cycles, before the final extension at 72°C for 10 min. At the end of the reaction, a 10-µL PCR product was used for 1% agarose gel electrophoresis.

RESULTS

In this study, the most common colony losses occurred in March, April, and June at rates of 38.1%, 21%, and 13.1%, respectively. A single *Varroa destructor* infection was detected in 21 samples. *Nosema* was detected in 10 samples and co-infection with both *Nosema* and *Varroa* was observed in eight samples *(Table 2, Table 3)*. *Tropilaelaps spp.* were not identified.

The results of this study also showed that DWV was the most prevalent honey bee virus affecting colony losses in Turkish apiaries as it was found in 44.7% (34/76) of the samples, followed in descending order of prevalence by ABPV, BQCV, SBV, CBPV, and IAPV with prevalence rates of 35.5% (27/76), 28.9% (22/76), 22.3% (17/76), 18.4% 14/76), and 6.5% (5/76), respectively *(Table 2, Table 3)*. KBV was not found in any of the samples collected from apiaries.

Mixed viral infections were also detected in our study *(Fig. 2)*, and most of the Turkish colonies were positively diagnosed with two viruses (19/76). Furthermore, three-

Table 2. *The provinces distributions of samples and the numbers of pathogens*

Provinces	*SMP	Varroa	Nosema spp.	IAPV	DWV	SBV	ABPV	BQCV	CBPV
Adana	9	4	1	5	1	0	2	2	1
Aydın	12	3	4	0	4	2	2	3	2
Bursa	3	1	0	0	2	0	0	0	1
İzmir	33	14	6	0	19	7	14	11	6
Kütahya	2	1	0	0	0	0	0	1	1
Manisa	2	0	1	0	0	0	0	0	0
Muğla	15	7	6	0	8	8	9	5	3
TOTAL	76	29	18	5	34	17	27	22	14

** **SMP:** Samples*

Table 3. *The monthly distributions of samples sent with the complaint of colony losses and the numbers of pathogens*

Months	*CL	Varroa	Nosema spp	IAPV	DWV	SBV	ABPV	BQCV	CBPV
March	29	6	6	5	10	0	4	6	4
April	16	6	7	0	8	6	7	3	2
June	10	5	3	0	3	6	7	6	5
October	9	6	1	0	6	0	6	4	2
November	5	3	0	0	4	1	1	1	0
September	4	2	1	0	2	2	0	2	1
May	2	1	0	0	0	2	2	0	0
February	1	0	0	0	1	0	0	0	0
TOTAL	76	29	18	5	34	17	27	22	14

***CL:** Number of colony losses*

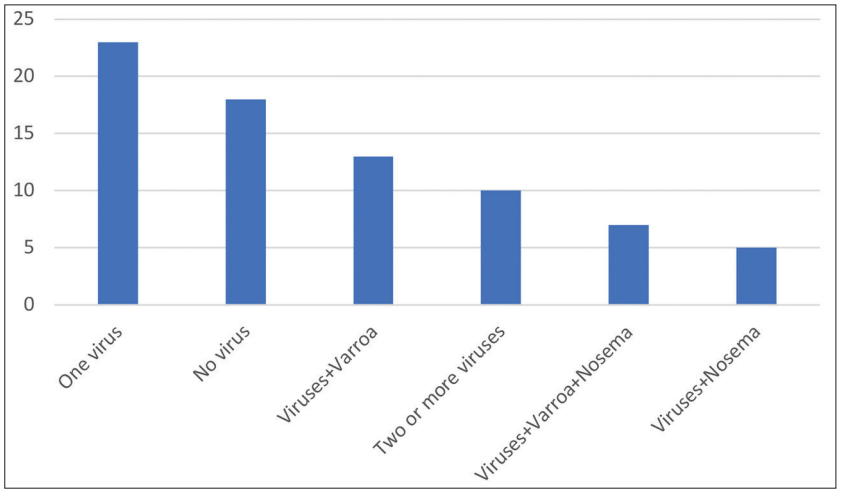

Fig 2. Mixed infections identified in Turkish apiaries affecting colony losses

virus infections were detected in 8 out of 76 samples, four-virus infections in 7 out of 76, while a five-virus infection was detected in one colony. In some apiaries, mixed viral infection was detected simultaneously with other parasitic infestations. A single virus was detected in 22 apiaries and no virus was detected in 19 apiaries.

Varroa, Nosema and viruses were co-occuring in some colonies. Parasitic infestation was found in apiaries that were positive for IAPV, DWV, SBV, ABPV, BQCV and CBPV at rates of 80%, 59.4%, 70.5%, 70.3%, 86.3%, 57.1% respectively. *Nosema* was detected in three, *Varroa* in four, and both *Nosema* and *Varroa* simultaneously in one of specimens without any detected virus.

DISCUSSION

In past years, bacteria, viruses, microsporidia, and parasites have all been reported to play an important role in colony losses [4,5,28,29]. Honey bee losses can be caused by either biotic or abiotic agents [10]. In this study, the presence of pathogens affecting colony losses were investigated in Turkish apiaries.

Most of the viruses identified in this study were RNA viruses; they were DWV, ABPV, BQCV, SBV, CBPV, IAPV. The general pattern of high DWV prevalence in our study is consistent with other published data concerning this matter [30,31]. The presence of DWV has been reported in apiaries with collapsed colonies in the Hatay province of Turkey [25]. ABPV was the second-most widespread bee virus in apiaries with colony losses in our study. In the previous studies from randomly sampled colonies in Turkey, ABPV was not found in the Black Sea Region [32] and it was determined at low incidence in the Aegean Region [33,34]. ABPV is one of three viruses, along with DWV and CBPV, that ANSES emphasizes in colony losses. The presence of DWV and ABPV in the current study are consistent with the ANSES report and the German bee-monitoring project findings [29]. BQCV is one of the most commonly detected bee viruses in Turkish colonies [35], and BQCV prevalence in the current study is consistent with previous studies in Turkey [32,34]. In contrast to this study, BQCV was the most frequently detected virus in Belgium Colony Collapse Disorders (CCD) [36]. CBPV is one of the most important viral agents causing colony losses and overt clinical signs. CBPV was detected in seven of 28 (25%) samples from the Black Sea region in Turkey [32] but other studies showed CBPV to be relatively rare in most provinces [34,37]. Cox-Foster et al.[4] showed a positive correlation between CCD and IAPV in their results. In this study, we detected IAPV in five different apiaries in just

one province (Adana), and it was identified simultaneously with other pathogens like *Varroa* and/or *Nosema*. These additional pathogens were detected in four apiaries, while IAPV alone was detected in one apiary, which may indicate that honey bee losses in the province of Adana may be due to IAPV. KBV is thought to be exotic for Europe [17] and has not been detected in previous studies by Cagirgan [34] in the Aegean Region of Turkey. SBV is one of the most widely distributed honey bee viruses. This virus can infect both larvae and adult stages of honey bees, but larvae are far more susceptible than adult bees. Worker bees play an important role in the transmission of this disease to the colonies [22]. In our study, SBV was only examined in adult bees. The Turkish genotype of SBV has been reported in colonies of *Apis mellifera* in Turkey [38]. It was previously reported in 2.7% of 111 colonies from different apiaries in seven provinces of the Aegean Region [34]. In this study, the prevalence of SBV in apiaries with colony losses was found to be higher than in the study conducted using the random sampling method by Cagirgan [34].

Honeybee colonies are commonly infected simultaneously by many viruses, often without exhibiting overt signs [39]. Mixed viral infections were detected in honeybee samples, but these simultaneous infections consisted of various pathogens as detailed in *Fig. 2*. These results are consistent with those obtained by Van Engelsdorp et al.[40], where a higher percentage of CCD colonies were co-infected with a greater number of disease agents than control colonies. Our mixed viral infection ratio, which was 46.1% (35/76), may indicate the presence of CCD in this study, but in most cases, mixed viral infections were observed simultaneously with *Varroa* and *Nosema* (25/35). There are multiple factors that can lead to colony losses, depending on the pathogen, pathogen titers and quality of nutrition. We were unable to obtain data about the quality of nutrition.

Varroa and *Nosema* were found in samples with rates of prevalence of 38.1% and 23.6%, respectively. *Varroa* is a major problem in beekeeping not only in Turkey, but across the world [41]. It was previously reported in 55% of 394 apiaries from seven provinces of the Aegean Region in Turkey [33]. *Varroa* is considered the main factor in winter colony losses according to Topalska et al.[42] and Van Engelsdorp et al.[43]. However, the honeybee losses in our study frequently occurred in spring. In Turkey, the presence of *Nosema* was confirmed earlier [44,45]. It has been reported that colony losses are caused by *Nosema* in the Hatay overwintering region and Southeastern Marmara [45]. Francis et al.[27] reported that viral titers will rise in honeybee populations from spring to autumn. *Nosema* peaked in the period between March to June and was detected in 18 colonies. On the other hand, the lower levels of *Nosema* detected in autumn could be due to the possible application of antifungal treatments [46]. When we compared our results with other Turkish colonies without colony losses [20,47], we found that their *Nosema spp.* levels were approximately equal to levels determined to affect colony losses in our study. Although *Nosema spp.* has been associated with CCD [4], according to a 5-year German study, there is no correlation between *Nosema* and honeybee losses [48]. Lack of correlation between *Nosema* and honeybee losses demonstrates the importance of mixed viral infections in our study. Therefore, mixed viral infections may be significant for honeybee losses in Turkish apiaries.

Many studies indicate that there is no single pathogen associated with colony losses [40,49]. However, in our study, viral agents like CBPV, BQCV, DWV, ABPV, SBV, parasites like *Varroa spp.* and microsporidia like *Nosema spp.* have been identified individually in some apiaries. Virus titers and excessive parasitic infestation may lead to individual colony losses in apiaries [50]. Moreover, many of the colony losses in early spring may be explained by the fact that colonies are weak and vulnerable at the end of winter. As use of pesticides is another cause for loss, colony losses could also be due to pesticides in apiaries where no viral or parasitic agent was detected. There is no up-to-date proof regarding the effect that simultaneous viral infections could have on the health of honeybees. Therefore, new research is required since the contribution of mixed viral infections to honeybee losses without any stress factors such as poor nutrition or parasites should be investigated further.

In conclusion, there are multiple factors that can lead to honeybee losses. Viruses in particular play a major role in declining colonies, and are a likely contributor to honeybee deaths in Turkey.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Statement of Author Contributions

Methodology: A. A. Çağırgan, K. Pekmez, M. Kaplan, B. Ozkan, H. Yeşilöz, F. Arslan, H. Yeşilöz; Sample preparation: A. A. Çağırgan, K. Pekmez, M. Kaplan, B. Ozkan, H. Yeşilöz, F. Arslan; Investigation: A. A. Çağırgan, K. Pekmez, M. Kaplan, H. Yeşilöz, A. Beyazıt; Data evalution: G. Kalaycı, A. A. Çağırgan, A. Beyazıt; Writing: A. A. Çağırgan

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Research Article

Roles of Histidine Kinase Gene *yycG* in the Pathogenicity of *Listeria monocytogenes*

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Abstract

As an opportunistic foodborne pathogen, *Listeria monocytogenes* could successfully switch self-characteristics in response to environmental conditions. In *L. monocytogenes,* sixteen two-component systems (TCSs) have been found to contribute the bacteria to sensing and adapting to various conditions. Our previous genomic study showed that mutation was observed in the histidine kinase gene *yycG* between Lm850658 and M7 among the sixteen TCSs. The YycFG TCS was found playing important roles in many other Gram-positive bacteria. While the roles of YycFG TCS in *L. monocytogenes* remain poorly known. In this study, we aimed to determine whether *yycG* play roles in pathogenicity of *L. monocytogenes*. We created a histidine kinase gene *yycG* deletion strain (Δ*yycG)* based on the wild type strain Lm850658 and a complemental strain (CΔ*yycG)*. We found the *yycG* deletion significantly impaired the growth ability and mobility, but enhanced the hemolysis ability *in vitro.* In addition, infection assays on cell and mice model showed that Δ*yycG* exhibited significantly defected in infection ability and virulence. All these phenotypes of the Δ*yycG* could be reversed largely to the levels of the wild type strain Lm850658 by gene complementation. Cell wall-associated and secreted protein analysis showed that the secreted content of Δ*yycG* was significantly increased. And western blotting revealed that Internalin protein B (InlB) and Listerolysin O (LLO) was markedly increased in the secreted fractions of Δ*yycG*, which might be responsible for decreased adhesion and invasion ability and increased hemolytic activity, respectively. Overall, we found the histidine kinase *yycG* played important roles in pathogenicity of *L. monocytogenes* for the first time. Further investigation is needed to explore how the YycFG TCS modulates the growth, mobility, cell surface proteins translocation and virulence.

Keywords: *Listeria monocytogenes, Two-component system, Histidine kinase yycG, Pathogenicity*

Listeria monocytogenes'in Patojenitesinde Histidin Kinaz Geni *yycG*'nin Rolü

Öz

Gıda kaynaklı fırsatçı bir patojen olan *Listeria monocytogenes,* çevresel koşullara tepki olarak kendi özelliklerini başarılı bir şekilde değiştirebilir. *L. monocytogenes*'te, onaltı adet iki bileşenli sistemin (TCS) bakterilerin çeşitli durumları algılamasına ve adaptasyonuna katkıda bulunduğu belirlenmiştir. Önceki genomik çalışmamız, on altı TCS'den Lm850658 ve M7 arasındaki histidin kinaz gen *yycG*'de mutasyon gözlendiğini göstermiştir. YycFG TCS'nin diğer birçok Gram pozitif bakteride önemli roller oynadığı belirlenmiştir. Oysa *L. monocytogenes*'teki YycFG TCS'nin rolü hala tam olarak bilinmemektedir. Bu çalışmada, *yycG*'nin L. monositogenes'in patojenisitesinde rol oynayıp oynamadığını belirlemeyi amaçladık. Yaban tipi Lm850658 suşunu ve tamamlayıcı bir suşu (C*ΔyycG)* temel alan bir histidin kinaz geni *yycG* silme suşu (*ΔyycG)* oluşturduk. YycG'nin silinmesinin büyüme kabiliyetini ve hareketliliğini önemli ölçüde bozduğunu, ancak in vitro hemoliz yeteneğini artırdığını bulduk. Ek olarak, hücre ve fare modelindeki enfeksiyon deneyleri, Δ*yycG*'nin enfeksiyon yeteneği ve virülansta önemli ölçüde kusurlu olduğunu gösterdi. Δ*yycG*'nin tüm bu fenotipleri, gen tamamlaması ile büyük ölçüde yaban tipi Lm850658 suşunun düzeyine tersine çevrilebilir. Hücre duvarı ilişkili ve salgılanan proteinler analizi, Δ*yycG*'nin salgılanan miktarının önemli ölçüde arttığını gösterdi. Western blot analizi, Δ*yycG*'nin salgılanan fraksiyonlarında; sırasıyla azalmış yapışma ve invazyon yeteneğinden ve artmış hemolitik aktiviteden sorumlu olabilecek Internalin protein B (InlB) ve Listerolysin O'nun (LLO) belirgin şekilde arttığını ortaya koydu. Histidin kinaz *yycG*'nin *L. monocytogenes* patojenitesinde önemli roller oynadığı ilk kez belirlendi. YycFG TCS'nin büyümeyi, hareketliliği, hücre yüzeyi proteinlerinin translokasyonunu ve virülansı nasıl değiştirdiğini belirlemek için ileri araştırmalara ihtiyaç vardır.

Anahtar sözcükler: *Listeria monocytogenes, İki bileşenli sistem, Histidin kinaz yycG, Patojenite*

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INTRODUCTION

Listeria monocytogenes is a non-spore forming Gram positive microorganism which contaminates food and has been frequently associated to foodborne disease. In humans, *L. monocytogenes* can cause listeriosis, a disease that ranges from febrile gastroenteritis to a more severe, invasive disease such as meningitis and septicemia [1,2]. *L. monocytogenes* infection cycle includes adhesion and invasion into host cells, escape from phagocytic or internalized vacuoles, intracellular multiplication, and cell to cell spread [1,3]. The major virulent factors mediate *L. monocytogenes* entry and survival in host cells are secreted proteins which are translocated to the bacterial surface. Among these virulent factors, internalins InlA and InlB mediate *L. monocytogenes* entry into intestinal epithelial cells or other types of cells via E-cadherin and c-Met, respectively [4]. We previous found that failed anchoring on cell surface of InlB was associated with the low-virulent phenotype of *L. monocytogenes* strain M7 [5]. Indicating that correction anchoring of the surface proteins is quite necessary for the function of these virulent factors. In Gram-positive bacteria like *L. monocytogenes*, the majority of secreted proteins are first translocated across the cytoplasmic membrane and then delivered to membrane-anchored, cell-wall anchored or extracellular milieu under suitable environments [6,7]. Anchoring of these secreted proteins were affected by complicated conditions, including ionic concentration, pH, redox potential and post-translocation modification [8-10].

Two-component regulatory signaling systems (TCSs) are used by most prokaryotic organisms to sense and adapt to various stress conditions encountered in nature, during food processing and infection [11]. A typical TCS is usually constituted of a transmembrane sensor histidine kinase and a cognate cytoplasmic response regulator [11]. In the first sequenced genome of *L. monocytogenes* EGDe, 16 TCSs have been identified [12]. These TCSs are also found in the genomes of the virulent strain Lm850658 and avirulent strain M7, which were sequenced by us. But difference was only observed in *yycG* of the YycFG TCS between Lm850658 and M7. YycFG TCSs are prevalent distribution in other Gram-positive bacteria, which share similar sequences and operon structures that contain 3-6 genes [13]. Previous studies showed that the YycFG TCS is quite important in those Gram-positive bacteria, since it plays essentially and regulatory roles in metabolism, cell division, cell wall synthesis, autolysis, biofilm formation and virulence [13-18]. While the roles of YycFG TCS in *L. monocytogenes* remain poorly known. Up to date, only Pontinen A. et al. showed that the histidine kinase YycG was involved in the growth of *L. monocytogenes* EGDe at low temperatures [19]. Whether YycFG TCS plays other roles in *L. monocytogenes* remains to be explore.

This study was aimed to determine whether *yycG* contribute to the virulence of *L. monocytogenes*. Therefore, we created a histidine kinase gene *yycG* deletion mutant using homologous recombination and a complemental strain. We found the *yycG* deletion significantly impaired the growth ability and mobility, but enhanced the hemolysis ability *in vitro*. In addition, infection assays on cell and mice model showed that the deletion of *yycG* in Lm850658 significantly defected the infection ability and virulence. Taken together, we found the histidine kinase *yycG* played important roles in pathogenicity of *L. monocytogenes* for the first time.

MATERIAL and METHODS

Bacterial Strains, Plasmids, Cells and Culture Conditions

Listeria monocytogenes virulent strain Lm850658 (serovar 4a) was used as the wild-type strain. *Escherichia coli* DH5α was employed as the host strain for shuttle vector pKSV7 and expression plasmid pIMK2. *L. monocytogenes* strains were cultured with brain heart infusion medium (BHI, Oxoid, Basingstoke, U.K.) and *E. coli* DH5α was cultured with Luria-Bertani (LB, Oxoid) medium at 37°C. Stock solutions of ampicillin (50 mg/mL), gentamycin (50 mg/mL), kanamycin (50 mg/mL) and chloramphenicol (10 mg/mL) (Sangong Biotech, Shanghai, China) were added to media at required concentrations. Macrophages RAW264.7 and epithelial cells Caco-2 (National Infrastructure of Cell Line Resource, Beijing, China) were cultured at 37°C and 5% CO_2 in complete RPMI 1640 medium (Gibco, New York, U.S.A.) containing 10% fetal bovine serum (FBS) (Gibco).

Plasmids Construction

The *yycG* gene fragment and the *yycG* homoarms were amplified from the genomic DNA of *L. monocytogenes* Lm850658 with oligonucleotide primers were synthesized by BGI-tech (Wuhan, China) *(Table 1)*. PCR fragments and plasmids were purified using the DNA Gel Extraction Kit and Unit-10 plasmid Extraction Kit (Sangong Biotech), respectively. Plasmids pIMK2 and pKSV7 were linearized with restriction enzyme *BamH*I (New England Biolabs, Massachusetts, U.S.A.) and then gel-purified. Purified PCR fragments and plasmid fragments were ligated by recombinase Exnase II with the One Step Clone Kit (Vazyme Biotech, Nanjing, China) as the manufacturer's instructions to produce recombinant plasmids (pIMK2-*yycG* and pKSV7-*yycG* homoarm). The ligation products were transformed into competent cells DH5α and positive clones were then confirmed by PCR and sequencing.

Construction of yycG Deletion and Complement Strains

A homologous recombination strategy was used for construction of *yycG* deletion mutant in *L. monocytogenes* wild-type strain Lm850658 according to the protocols [20]. The recombinant plasmids pKSV7-*yycG* homoarm were electroporated into *L. monocytogenes* competent cells. Transformants were grown at a non-permissive temperature (41°C) in BHI containing chloramphenicol (10 μg/mL) to

Table 1. *Primers used for deletion and complement mutants construction*

Primers	Sequences (5'-3')
yycG-A	CATGCAGGTCGACTCTAGAGATGTAACGAAACCATTCAGTAAC
yycG-B	AGCGAAATCCTGTTTTTTTCATCATAAGTCTTTCTACTCCTATTCG
yycG-C	ATGATGAAAAAAACAGGATTTCGCT
yycG-D	ATTCGAGCTCGGTACCCGGGGTCAGCTTGCTTTCATTCTTTTTA
yycG-E	ATGATGGCGATGAAGCGTTAGA
yycG-CF	GAAGGAGAGTGAAACCCATGGAAAAGATGCATAAAATGAGATTTTTTCAGTCTGTACA
yycG-CR	CGAATTCCTGCAGCCCGGGGTCATTCCCAATCATCCTCCGGT

promote chromosomal integration and recombination. The recombinants were passaged, in succession, in BHI without antibiotic at a permissive temperature (30°C) to enable plasmid to be eliminated. The *yycG* gene deletion strain Lm850658-Δ*yycG* was identified by PCR and then confirmed by sequencing. For the complement strain, recombinant plasmids pIMK2-*yycG* were electroporated into Lm850658-Δ*yycG* competent cells.

Hemolysis Assay

Listeriolysin O (LLO) was tested for hemolytic activity as described [21]. Aliquots of 950 µL 5% sheep red blood cells suspension in 10 mM phosphate buffered saline (PBS) were added 50 µL filtered overnight culture supernatants with 0.22 µm filter (Millipore, Massachusetts, U.S.A.), and 50 µL BHI with or without 10% triton-X-100 were set as negative and positive control, respectively. After incubating at 37°C for 6 h, samples were centrifugated at 1.000 g for 5 min and the optical density values of the supernatants (200 µL per well) were detected by spectrophotometer at 550 nm in 96 microwell plate.

Adhesion and Invasion Assays in Caco-2 Cells

Overnight cultures were harvested by centrifugation (5.000 g for 10 min), resuspended in 10 mM PBS (pH 7.4) and adjusted the optical density at the wavelength of 600 nm ($OD_{600\ nm}$) to 0.25. The epithelial cell line Caco-2 was grown at 37°C and 5% CO_2 for 18-24 h to confluence (about 2×10^5) in 12-well plates (Corning, New York, U.S.A.) and infected with *L. monocytogenes* at multiplicity of infection (MOI) of 10:1 for 1 h. For adhesion assay, cells were lysed after three times of washing with PBS. For invasion assay, cells were washed with PBS after 1 h infection and incubated for an additional 1 h in RPMI 1640 medium containing 10% FBS and 50 µg/mL gentamycin. At the indicated times, the cells were lysed and 10-fold diluted for plating on BHI agar. The agar plates were incubated overnight at 37°C for colony counting. Adhesion rate was expressed as the percentage of recovered colonies to those inoculated; while invasion rate was calculated as the percentage of recovered colonies after gentamycin treatment to those inoculated.

Survival in Macrophage RAW264.7

Cells were infected with resuspended bacteria as above (MOI =10:1) at 37°C and 5% CO_2 for 1 h. Extracellular bacteria were killed with additional 1 h incubation in the presence of 50 µg/mL gentamycin. Cells were lysed with ice-cold disitilled water after three times of washing with PBS and diluted appropriately for plating on BHI agar plates for colony counting to calculate the initial intracellular bacteria. To count intracellular survived bacteria, cells were cultured for another 3 h in the presence of 5 µg/mL gentamycin and then lysed with ice-cold distilled water after three times of washing and diluted appropriately for plate counting. Survival rate was calculated for each strain by dividing the final recovered colony counts with those initial intracellular counts.

Actin-tail Formation in Caco-2 and Macrophage Cells

Actin-tail formation assay was conducted as our previous research [22]. Briefly, the Caco-2 and RAW264.7 cells were seeded in 24-well plate and cultured in RPMI 1640 medium containing 10% FBS. Overnight bacterial cultures were harvested and adjusted $OD_{600\ nm}$ at 0.25. Cells were infected at MOI of 10:1 at 37°C and 5% CO_2 for 1 h. Extracellular bacteria were killed with 50 µg/mL gentamycin for 1 h and then incubated for additional 5 h. Cells was washed gently with 10 mM PBS, fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. The bacteria were probed with *L. monocytogenes* polyclonal antibodies which was produced by our laboratory for 1 h at 37°C, washed twice with PBS and stained with Alexa Fluor 488 conjugated donkey anti-rabbit antibody (Thermo Fisher Scientific, Massachusetts, U.S.A.) for 1 h at 37°C. F-actin was then stained with 6.6 µM phalloidin-Alexa Fluor 568 (Thermo Fisher Scientific). The 6-diamidine-2-phenylindole (DAPI, Thermo Fisher Scientific) was used to stain the nuclei. Actin tails recruited by the bacteria were visualized by confocal microscope (Olympus FV 1000, Tokyo, Japan) and indicated by arrows.

Virulence in Mice Model

The assay was conducted as our previous research [23]. Four-weeks age female ICR mice, twenty per group, were acclimatized for three days in a standard class II laboratory animal facility. Overnight cultures were treated as above with adjusted $OD_{600\ nm}$ at 0.6. Mice were inoculated intraperitoneally with 2×10^5 bacteria. At 24 and 48 h post

infection (pi), mice (six per group) were euthanized, liver and spleen samples were homogenated, and diluted appropriately for plating counting on BHI agar plates. The rest mice were observed for mortalities twice a day for 7 days. Animal experiments were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

Surface Proteins Analysis

Surface proteins analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting was conducted as our previous research [23]. Bacteria were cultured in 100 mL BHI medium at 37°C for 12 h, and then harvested by centrifugation (15.000 g, 10 min). The supernatant samples were filtered through 0.22 μm filter (Millipore) and proteins were precipitated with 100% (w/v) trichloroacetic acid to a final concentration 10% (w/v) at 4°C. Cell wall associated proteins were extracted from bacterial pellet with 1% SDS (30 mg wet weight per ml of 1% SDS) at 37°C for 1 h. Protein samples were analyzed by 12% SDS-PAGE gel. Abundance of the secreted and surface-associated proteins InlA, InlB, and LLO were blotted and probed with respective polyclonal antibodies. The glyceraldehyde-phosphate dehydrogenase (GAPDH) was probed and set as loading control. All primary antibodies including anti-InlA, anti-InlB, anti-LLO and anti-GAPDH polyclonal antibodies were produced in our laboratory and the secondary antibody HRP-conjugated Goat anti-Rabbit IgG (D110058) was purchased from Sangong Biotech.

Statistical Analysis

All results were presented as mean ± SD of triplicate experiments and subjected to one-way analysis of variance (One-way ANOVA). Differences were considered as statistically significant at $P<0.05$. Notations of statistics marked as * and ** indicated the statistical significance with $P<0.05$ and $P<0.01$, respectively.

RESULTS

Our previous research showed that *yycG* of Lm850658 contains a mutant, which results in the 220th amino acid residue mutation in HAMP domain [22]. To clarify the role *yycG* in the pathogenicity of *L. monocytogenes*. We constructed a *yycG* in-frame deletion strain (Δ*yycG)* based on Lm850658 and a complemental strain (CΔ*yycG)* that express *yycG* with the integrated plasmid pIMK2. We found that the growth ability of mutant strain Δ*yycG* was significantly lower than that of the wild type strain Lm850658 in BHI broth *(Fig. 1-A)*. While the complemental strain CΔ*yycG* partially recovered the growth defect *(Fig. 1-A)*. The mobility of mutant strain Δ*yycG* was conspicuous smaller than that of the wild type and complemental strains *(Fig. 1-B)*. But the hemolytic ability of mutant strain Δ*yycG* was significantly stronger than that of the wild type and complemental strains as showed in 5% sheep red blood cells suspension *(Fig. 1-C)*. Infection assay on epithelial cells Caco-2 cells showed that the adhesion rate (1.07%±0.22%) and invasion rate (0.08%±0.01%) of mutant strain Δ*yycG* were significantly lower than those of the wild type strain and the complemental strain, respectively ($P<0.01$) *(Fig. 2-A,B)*. Survival in macrophages RAW264.7 showed that the survival rate of the wild type was 123.33%±10.12% at 4 h pi, which was significantly higher than that of the mutant strain Δ*yycG* *(Fig. 2-C)*. And the complemental strain exhibited the similar survival ability as the wild type strain ($P<0.01$) *(Fig. 2-C)*. Actin tail formation assay showed

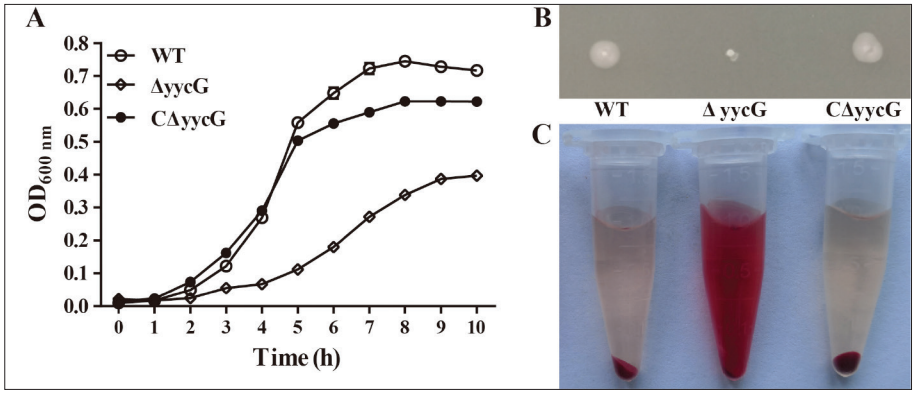

Fig 1. The *yycG* deletion strain Δ*yycG* exhibited defect in growth and mobility, but enhance in hemolysis, compared with the wild type strain Lm850658 (WT) and complemental strain CΔ*yycG*. Growth curve was made based on the OD_{600nm} values with 1 h interval in BHI broth at 37°C **(A)**. Mobility was tested on the soft agar plate (0.3% agar) under 30°C for 48 h **(B)**. Hemolysis assay was conducted in 5% sheep red blood cells suspension with the cultural supernatants at 37°C for 6 h **(C)**. The representative results were showed from at least three independent experiments

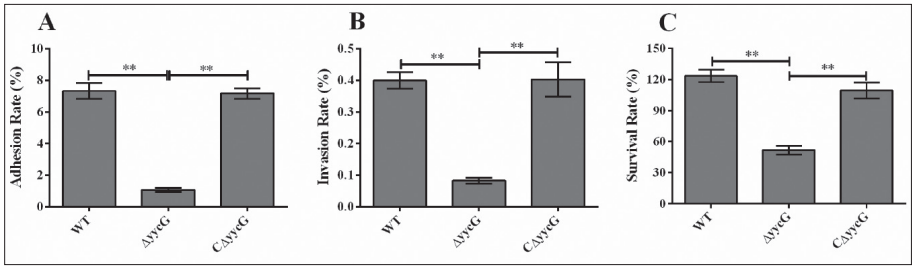

Fig 2. The *yycG* deletion significantly defected the infection ability to both epithelial cells Caco-2 and macrophages RAW264,7. Adhesion **(A)** and invasion **(B)** ability was conducted on epithelial cell Caco-2, and survival assay was conducted on macrophages RAW264.7 **(C)**. Data was presented as mean ± SD of three independent experiments and ** indicated the statistical significance with $P<0.01$

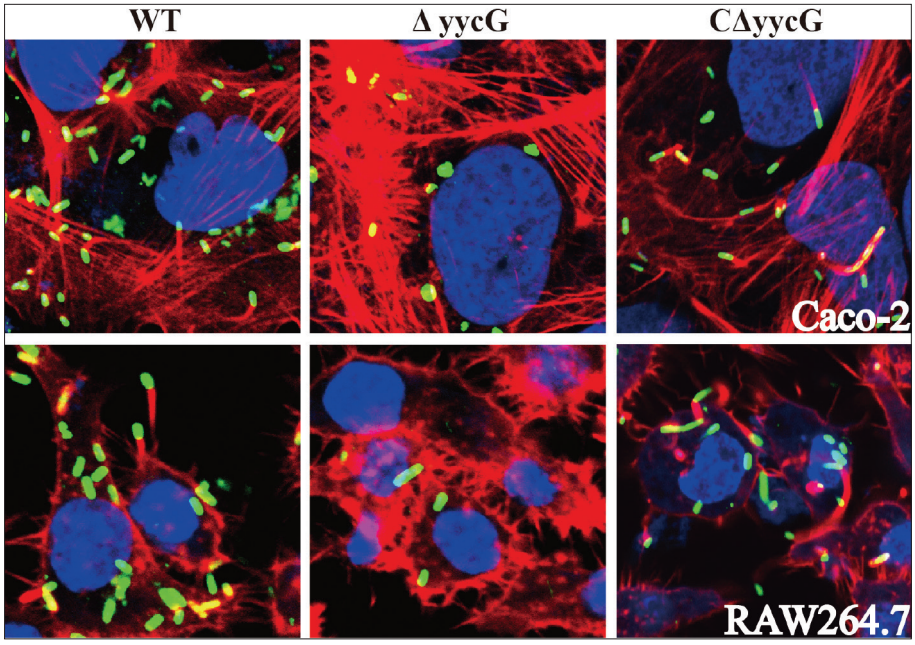

Fig 3. The *yycG* deletion significantly defected the actin tail formation ability to both epithelial cells Caco-2 (up) and macrophages RAW264,7 (down)

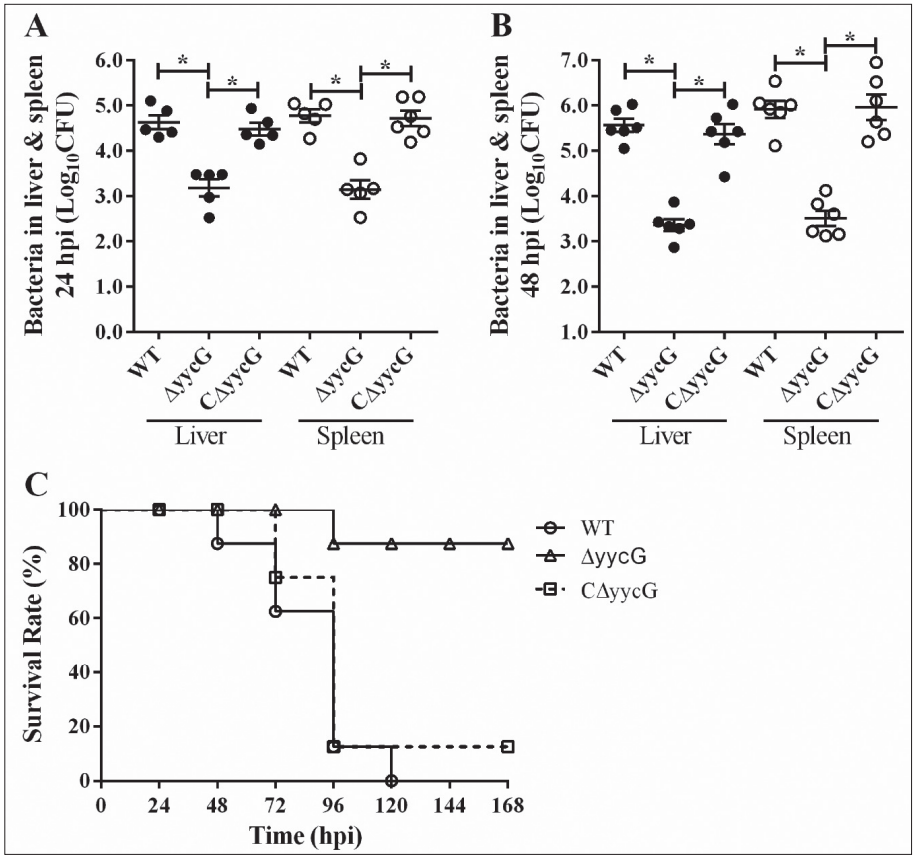

Fig 4. The *yycG* deletion significantly impaired the pathogenicity of *L. monocytogenes* on mice model. The *yycG* deletion significantly decreased bacteria load in the liver and spleen of infected mice at both 24 **(A)** and 48 **(B)** hr post infection (pi), and increased the mortality rate of infected mice **(C)**. Data was presented as mean ± SD, * indicated the statistical significance with $P<0.05$

that deletion of *yycG* almost absolutely abolished the cell-to-cell spread ability of Lm850658 in both epithelial cells Caco-2 and macrophages RAW264.7, and this phenotype was partially recovered in the complemental strain *(Fig. 3)*.

To evaluate the role of *yycG in vivo*, the intraperitoneal infection model showed that the bacterial load in liver and spleen samples of the mutant strain Δ*yycG* infected mice were significantly less than those of the wild type and complemental strains infected mice at 24 and 48 h pi ($P<0.05$) *(Fig. 4-A,B)*. All mice infected with the mutant strain Δ*yycG* remained alive before 96 hr pi, and only one (12.5%, 1/8) mouse died during the observation period, while the mice infected with wild type strain died within one week *(Fig. 4-C)*. Moreover, 87.5% (7/8) of mice infected

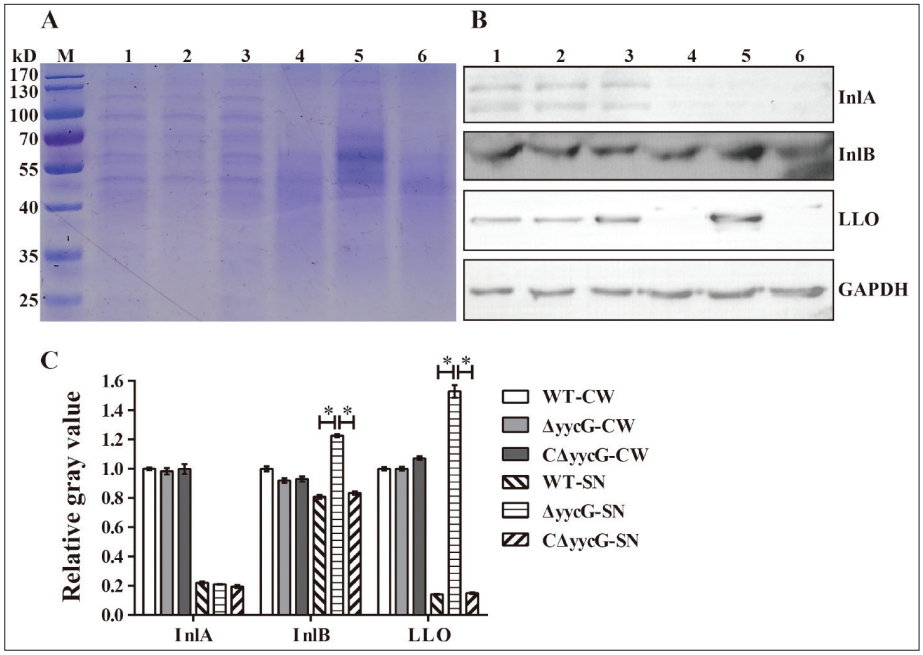

Fig 5. The *yycG* deletion significantly increased secretion of InlB and LLO. Cell surface extracted fractions (lane1, WT; lane 2, Δ*yycG*; lane 3, CΔ*yycG*) and secreted fractions (lane4, WT; lane 5, Δ*yycG*; lane 6, CΔ*yycG*) were analyzed by SDS-PAGE **(A)** and western blot **(B)**. The relative contents of InlA, InlB and LLO in different strains were analyzed by calculated the density of the specific bands **(C)**. * indicated the statistical significance with P<0.05

with the complemental strain died during the seven days observation period *(Fig 4-C)*. These results suggest that *yycG* deletion significantly weaken the pathogenicity of *L. monocytogenes*.

As pathogenicity of *L. monocytogenes* were mainly determined by the cell surface virulent factors. To explore the mechanisms that *yycG* affected the virulence of *L. monocytogenes*, we analyzed the bacterial surface associated virulent factors of wild type and *yycG* deletion strain. Our data showed that the content of some proteins extracted from the cell surface of the mutant strain Δ*yycG* were obvious less than those of the wild type and *yycG* complemental strain *(Fig. 5-A)*. While opposite pattern was observed in cultural supernatant samples of these strains *(Fig. 5-A)*. Western blotting analysis showed that content of InlB and LLO of mutant strain Δ*yycG* were significant more than those of wild type and *yycG* complemental strain in secreted fraction (P<0.05), while no significant difference was observed in the cell surface extraction *(Fig. 5-B,C)*. And the distribution of InlA remained unchanged among the three strains in cell surface extraction and secreted fraction *(Fig. 5-B,C)*.

DISCUSSION

Listeria monocytogenes is an environmental saprotroph that becomes a pathogen following ingestion by mammalian hosts [2]. Yet, not all *L. monocytogenes* strains have the same pathogenicity because transition from environmental organism to pathogen requires significant changes in expression and translocation of virulent factors. These changes happened upon bacteria sensing subtle changes of the environments. TCSs play important roles in adapting to arduous environmental stress factors. YycFG as one of the most highly conserved TCSs in Gram-positive bacteria, exhibits diverse functions in different bacteria. The *yycG* has been shown to response to low temperature, but not to heat, acid, alkali, osmotic, ethanol and oxidative stresses [19,24]. In this study, we found *yycG* deletion significant impaired the growth ability and mobility, but enhanced the hemolysis ability *in vitro (Fig. 1)*, indicating multiple roles for the histidine kinase YycG in *L. monocytogenes*.

Further study showed that *yycG* in *L. monocytogenes* Lm850658 also plays roles in adhesion and invasion to epithelial cells and survival in macrophage RAW264.7 *(Fig. 2)*. Combination with actin tail formation assay and animal assay, we found that *yycG* deletion significantly affect the pathogenicity of Lm850658 *(Fig. 3, Fig. 4)*. Our findings expanding the roles of *yycG* in strain EGDe [12,19]. This might be explained by individual difference between Lm850658 and EGDe, as strain-specific gene function could be observed in other *L. monocytogenes* strains [25]. That is to say YycFG TCS might play different roles in various strains. Although Lm850658 and EGDe were belong to the same species, but they were clustered into different phylogenetic lineage that exhibited different characteristics. In Lm850658, *yycG* deletion results in severe defects in growth, survival in host cells and pathogenicity, and *yycFG* double mutant was failed to obtain. These evidences support that YycFG TCS is essential for *L. monocytogenes*. Since the essential genes knocking out will result in severe defects or lethal effects. Moreover, the essential *yycG* of *Bacillus subtilis* could be deleted only in the background of containing an inducible plasmid that expressed *yycG* [18]. In *Bacillus subtilis*, YycG senses cell division and co-ordinates cell wall remodeling by controlling the transcription of genes for autolysins and their inhibitor [18]. As a regulator of cell wall metabolism, dysfunction of YycFG TCS results in growth defect, cell wall architecture disorder and even cell death via multiple YycFG regulated genes [26].

The abnormal distribution of InlB and LLO in *yycG* deletion strain might be result from disturbing the cell wall remodeling. Since the anchoring of InlB onto cell surface need lipoteichoic acid (LTA) and suitable cationic conditions [27]. And the secretion and maturation of LLO was also associated with the environments between cell membrane and cell wall [28]. Furthermore, increase of the dissociated InlB and LLO from the secreted fraction do not seem to be benefit for the pathogenicity of *L. monocytogenes*, as demonstrated by *in vitro* and *in vivo* infection assays *(Fig. 2, 3* and *4)*. Previous research showed that exogenous InlB could also activate the c-Met signaling [29,30], which might compete with the cell surface InlB for bacteria invasion. In this way, secreted InlB dissociated from the bacterial surface might inhibit the adhesion and invasion of *L. monocytogenes* to host cells.

Listerolysin O is a versatile virulent factor involving in several infection progresses of *L. monocytogenes* [28]. Extracellular LLO is sufficient to direct bacterial internalization into HepG2 cells via activating tyrosine kinases and their downstream signaling [31]. Once entry into the cytosol, LLO could induce mitochondrial fragmentation, endoplasmic reticulum stress, unfolded protein response, protein degradation, inflammasome activation and cell-to-cell spread [28]. In the phagosome, LLO could perforate the membrane of phagosomes in graded, manage reactive oxygen species, establish chronic infection and induce autophagy [28]. But most of the functions of LLO appear to be by-products of either pore formation or entry of *L. monocytogenes* into the cytosol [28,32]. Since LLO is a multiple function factor whose expression, translocation, activation and even degradation was tightly regulated [28,33]. It is not difficult to find that un-controlled LLO is not benefit for the subtle intracellular lifecycle of *L. monocytogenes* as observed in previous reports [34,35] and in this study.

Taken together, we demonstrated that *yycG* played important roles in growth and pathogenicity of *L. monocytogenes* Lm850658. Further study might focus on the mechanisms of the *yycG* involved in the growth and pathogenicity of Lm850658 and the roles of the whole YycFG TCS in *L. monocytogenes*.

Statement of Author Contributions

XWF, WH and CF designed the project, conducted most of the experiments and drafted the manuscript. XWF, WH, YZ, CW, QPL, HW, XYL, YFG and CF were involved in study design and animal experiments. CF and YYY supervised the study and critically read the manuscript.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest: All authors declare no conflict of interest.

Research involving Human Participants and/or Animals: This article does not contain any studies with human participants. Mice used for virulent assays were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

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Research Article

Meta Analysis of Allele and Genotype Frequency of Growth Hormone (*b*GH) Gene *Alu*I Polymorphism, Which is Effective on Milk Yield in Holstein Cattle [1]

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Abstract

There are studies investigating the relationship between *b*GH gene *Alu*I polymorphism and milk yield in bovine breeds. In the presented study, the relationship between different genotypes and lactation milk yields was determined by analyzing *b*GH-*Alu*I genotype and allele frequencies in Holstein breed by meta-analysis method. The study material consisted of studies investigating bGH-AluI polymorphisms that are effective on milk yield in Holstein breed. According to the determined criteria, allele and genotype frequencies of 4583 samples in 20 studies selected from 35 studies and milk yields of 932 samples in 6 studies in which milk yields were calculated according to genotypes were included in the meta analysis. In the study, meta analyzes LL, LV and VV genotypes and L and V alleles and lactation milk yields were performed for bGH gene. The study samples were proved to be unbiased with the Begg and Mazumdar Rank Correlations test and funnel plot. As a result of the study, high heterogeneity was determined between the investigated studies concerning all genotypes (LL, LV and VV) and allele frequencies (L and V), thus random effect model was used. Meta analyzes were done with Comprehensive Meta-Analysis Software (CMA). According to the random effect model, pooled ratios in LL, LV and VV genotypes are 0.710, 0.260 and 0.030; pooled ratios for L and V allele frequencies were calculated as 0.818 and 0.182 and were found statistically significant (P<0.001). In addition, mean milk yields in LL, LV and VV genotypes were calculated as 6.64, 6.96, 7.13 L x1000 according to meta-analysis and subgroup analysis results, but no significant difference was found (P=0.958). At the end of the study, closer results to the population parameter were obtained regarding the relationship between bGH-AluI polymorphism genotype and milk yield in Holstein cattle with meta-analysis.

Keywords: *AluI, bGH gene, Holstein, Meta analysis, Milk yield*

Holştayn Irkı Sığırlarda Süt Verimi Üzerine Etkili Büyüme Hormonu (*b*GH) Geni *Alu*I Polimorfizminin Allel ve Genotip Frekanslarının Meta Analizi

Öz

Sığır ırklarında *b*GH geni *Alu*I polimorfizmi süt verim ilişkilerinin araştırıldığı çalışmalar bulunmaktadır. Sunulan çalışmada Holstayn ırkında *b*GH-*Alu*I genotip ve allel frekanslarının meta analizi yöntemi ile analiz edilerek, farklı genotipler ve laktasyon süt verimleri arasındaki ilişki belirlenmiştir. Çalışma materyalini, Holştayn ırkında süt verimi üzerine etkili *bGH-Alu*I polimorfizmlerinin araştırıldığı çalışmalar oluşturmuştur. Belirlenen kriterlere göre 35 çalışma içerisinden seçilen 20 çalışmadaki toplam 4583 örneğin allel ve genotip frekansı ve seçilen çalışmalar içerisinde genotiplere göre süt verimlerin hesaplandığı 6 çalışmadaki 932 örneğin süt verimleri meta analizine dahil edilmiştir. Çalışmada bGH geni LL, LV ve VV genotipleri, L ve V allellerinin frekansları ve laktasyon süt verimleri için meta analizleri yapılmıştır. Çalışma örneklemlerinin yanlı olmadığı Begg ve Mazumdar Sıra Korelasyonları testi ve huni grafiği ile kanıtlanmıştır. Çalışma sonucunda, tüm genotiplerde (LL, LV ve VV) ve allel frekanslarında (L ve V) incelenen çalışmalar arasında yüksek heterojenite belirlenmiş, bu nedenle rassal etki modeli kullanılmıştır. Meta analizleri Comprehensive Meta-Analysis Software (CMA) ile yapılmıştır. Rassal etki modeline göre LL, LV ve VV genotiplerinde ortak frekanslar 0.710, 0.260 ve 0.030; L ve V allel frekansları için ortak frekanslar 0.818 ve 0.182 olarak hesaplanmış ve istatistiksel olarak önemli bulunmuştur (P<0.001). Ayrıca, LL, LV ve VV genotiplerinde ortalama süt verimleri meta analizi ve alt grup analizi sonuçlarına göre 6.64, 6.96, 7.13 L x1000 olarak hesaplanmış ancak anlamlı farklılık bulunmamıştır (P=0.958). Çalışma sonucunda yapılan meta analizi ile Holştayn ırkı sığırlarda bGH-AluI polimorfizminin genotip ve süt verimi arasındaki ilişkileri hakkında popülasyon parametresine daha yakın sonuçlar elde edilebilmiştir.

Anahtar sözcükler: *AluI, bGH geni, Holştayn, Meta analizi, Süt verimi*

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INTRODUCTION

Growth hormone secreted from the anterior pituitary gland and functioning in many different physiological processes such as milk yield, growth, fertility in farm animals is a protein-nature hormone with a single chain polypeptide structure consisting of 191 amino acids [1-3]. In cattle, the growth hormone (bGH) gene, localised in chromosome 19, is 1793 base pairs and consists of 5 exons and 4 introns [4]. Because it plays an important role in physiological processes, the bGH gene has been reported to be a potential candidate gene for growth and live weight gain, milk yield, fertility and embryo quality in farm animals [2]. Studies conducted by different researchers especially in terms of milk yield characteristics have revealed a relationship between different SNP regions in the bGH gene and milk yield characteristics [4-6].

In recent years, rapid developments in molecular technologies and molecular genetic applications have been used in the breeding and selection of livestock. When cost-benefit comparison is made between molecular genetic analysis methods, restriction fragment length polymorphism (RFLP) method has significant advantages. Bacterial restriction enzymes, (restriction endonuclease) are the enzymes that make double-strand breaks in DNA at specific sites by recognizing specific nucleotides of target genes on double-stranded DNA [7].

Restriction enzymes play a role in the defense mechanism of bacteria by breaking DNA outside the bacterial genome from specific nucleotide regions [8]. In RFLP studies, these specific enzymes produced by bacteria are isolated and used. Restriction enzymes used in RFLP studies are named using the names of the bacteria from which they were isolated [9]. The *Alu*I restriction enzyme used in the bGH-*Alu*I polymorphism studies selected in this study was obtained from *Arthrobacter luteus* [9].

There are many studies investigating bGH-*Alu*I polymorphism and the relationship between this polymorphism and milk yield characteristics in cattle [4,5,10]. Different results are found in many studies investigating the relationship between bGH-*Alu*I polymorphism and milk yield in different Holstein populations. Although different findings obtained from several studies on the same subject have been reviewed in reviews, meta-analysis method has been developed to combine the results and make them a common finding by using statistical methods. Meta-analysis is carried out to increase the precision and power of the parameter estimation by combining the results of the studies conducted with small samples to increase the sample width in a consistent and coherent manner [11].

Today, meta-analysis has found widespread use in many fields such as medicine, veterinary, biology, agriculture, psychology, education and its importance has increased gradually. In recent years, meta-analysis studies have also been found in the field of genetics [12-14].

In this study, it was aimed to determine the heterogeneity between the studies and calculate pooled ratios by analyzing the results concerning the genotype and allele frequencies obtained from the studies investigating bGH-*Alu*I polymorphism in Holstein breed cattle with the meta-analysis method.

According to the pooled genotype and allele frequency ratios calculated from the studies included in the meta-analysis, the bGH-*Alu*I polymorphism Hardy-Weinberg χ^2 test results for the entire population were calculated.

Additionally, meta-analysis and subgroup analyzes of the mean yield values of studies determining lactation milk yields according to genotypes were performed and milk yields were compared according to genotypes.

MATERIAL and METHODS

The literature to be included in the meta analysis in our study have been accessed by searching the PubMed, Web of Science and Google Scholar databases using the keywords "bGH gene", "*Alu*I restriction enzyme" and "Holştayn-Holstein". The study material consisted of 20 studies selected from 44 primitive studies conducted between 2002 and 2018 (See supplementary table for references). The criteria for inclusion in the meta analysis were use of Holstein cattle as animal material as well as *Alu*I restriction enzyme in genetic analysis, and determining genotype (LL, LV and VV) and allele (L and V) frequencies in terms of bGH-*Alu*I polymorphism. In addition, subgroup analyzes were performed according to the genotypes used for meta-analysis of the yield averages of 6 studies investigating the relationship between genotype and lactation milk yields in terms of bGH-*Alu*I polymorphism. After determining the studies that met the criteria for inclusion in the meta-analysis, Begg and Mazumdar Rank Correlation test was used to determine whether the study sample is unbiased. The heterogeneity of the effect sizes between studies was evaluated in order to decide the meta-analysis method (fixed/random effect model) to be used to combine the results of the study and calculate the pooled ratios. Cochran's Q test statistics, I^2 statistics and τ^2 statistics were calculated to determine the heterogeneity between studies, to determine the level of heterogeneity and to determine the true variance between studies respectively. The simplest and most common approach used to assess whether there is true heterogeneity between studies is the (k-1) degree of freedom Chi-Square heterogeneity test proposed by Cochran, which is known as the Q statistic and is calculated as follows:

$$Q = \sum_{i=1}^{k} w_i Y_i^2 - \frac{\left(\sum_{i=1}^{k} w_i Y_i\right)^2}{\sum_{i=1}^{k} w_i}$$

It is also referred to as the standard χ2 test in the literature. I^2 statistic can be used to find out to what extent the observed variance reflects the true variance in effect size. I^2 is calculated as follows:

$$I^2 = \left(\frac{Q - df}{Q}\right) \times \%100$$

τ^2 the Tau-square coefficient (τ^2) is the variance of the true effect size and is calculated as follows:

$$\hat{\tau}^2 = \begin{pmatrix} \dfrac{Q - (k-1)}{\sum w_i - \dfrac{\sum w_i^2}{\sum w_i}}, & Q > (k-1) \\ 0, & Q \leq (k-1) \end{pmatrix}$$

Effect size indicates the magnitude of the result of an exposure [12]. The random effect model (Der Simonian-Laird method) was used in the analyses because the effect sizes were heterogeneous among the studies. The random effect model takes into account the variance of studies both within and between studies and assumes differences in effect size among all studies. All of the meta analyzes in the study were done with Comprehensive Meta-Analysis Software (CMA).

The genotype and allele frequencies of the bGH-AluI polymorphism of the studies included in the meta analysis are given in *Table 1*.

Means of milk yield (x1000, Liter) in studies in which lactation milk yield are determined according to bGH-*Alu*I polymorphism genotypes are given in *Table 2*.

RESULTS

In the study, genotype and allele frequencies in terms of bGH-AluI polymorphism belonging to 4583 head Holstein cattle in 20 studies conducted between 2002-2018 were included in the meta-analysis. The ratios of the frequencies of the genotypes and alleles determined in these studies and the Hardy-Weinberg (HW) χ^2 test results of the populations are given in *Table 3*. Thus, it was determined that the populations of 9 studies in terms of investigated genotype were not in HW equilibrium (P<0.05).

A total of 4583 Holstein cattle examined in 20 studies were included in the meta-analysis of LL, LV and VV genotypes and L and V alleles in the present study. Cochran's Q, I^2 and τ^2 test statistics were calculated to evaluate heterogeneity between studies for all genotype and allele frequency ratios. According to the calculated I^2 (%) values, high hetero-geneity was detected in all genotype and allele frequency ratios.

Table 1. *Genotype and allele frequencies of studies included in meta analysis*

No	Study	Year	Country	n	Genotype Frequency			Allel Frequency	
					n(LL)	n(LV)	n(VV)	n(L)	n(V)
1	Dybus et al.	2002	Poland	1086	709	352	25	885	201
2	Biswas et al.	2002	India	33	24	8	1	28	5
3	Sørensen et al.	2002	Denmark	415	311	85	19	354	61
4	Kováks et al.	2006	Hungary	363	314	47	2	338	25
5	Zakizadeh et al.	2006	Iran	110	79	29	2	17	94
6	Pawar et al.	2007	India	55	30	22	3	41	14
7	Balogh et al.	2008	Hungary	22	18	4	0	20	2
8	Hradecká et al.	2008	Germany	315	288	27	0	301	14
9	Balogh et al.	2009	Hungary	307	247	57	3	275	32
10	Mohammadabadi et al.	2010	Iran	103	56	32	15	72	31
11	Misrianti et al.	2012	Indonesia	370	329	41	0	348	22
12	Heidari et al.	2012	Iran	100	78	21	1	88	12
13	Akyuz et al.	2013	Turkey	150	116	9	26	126	24
14	Hadi et al.	2015	Iran	150	59	92	0	104	47
15	Hartatik et al.[a]	2015	Indonesia	19	16	3	0	17	2
16	Hartatik et al.[b]	2015	Indonesia	43	34	9	0	39	4
17	Molee et al.	2015	Thailand	231	0	198	33	132	99
18	Ozdemir et al.	2017	Turkey	186	93	89	4	138	48
19	Sonmez et al.	2018	Turkey	115	47	56	12	75	40
20	Amiri et al.	2018	Tunisia	410	319	76	15	357	53
Total				**4583**	**3166**	**1256**	**161**	**3754**	**829**

Table 2. *Mean of milk yields (x1000, Liter) of bGH-AluI polymorphism genotypes*

No	Study	Year	LL		LV		VV	
			n	X±Sx	n	X±Sx	n	X±Sx
1	Kováks et al.	2006	314	10.01±1.48	47	10.33±1.59	2	10.16±1.31
2	Pawar et al.	2007	30	3.16±0.09	22	3.89±0.08	3	3.80±0.05
3	Hadi et al.	2015	59	9.32±0.54	92	8.98±0.52	-	-
4	Hartatik et al.[a]	2015	16	4.00±1.56	3	5.19±0.23	-	-
	Hartatik et al.[b]	2015	34	5.66±1.83	9	5.79±1.49	-	-
5	Ozdemir et al.	2017	93	7.02±0.39	89	7.16±0.37	4	7.01±0.66
6	Sönmez et al.	2018	47	7.27±0.16	56	7.35±0.15	12	7.84±0.27

Table 3. *The studies included in the meta analysis and genotype and allele ratios of the herds examined*

Study	Genotype			Allel		HW χ^2 Values	P Values
	LL	LV	VV	L	V		
Dybus et al.	0.653	0.324	0.023	0.815	0.185	6.029	**P<0.05**
Biswas et al.	0.730	0.240	0.030	0.850	0.150	0.108	P>0.05
Sørensen et al.	0.749	0.205	0.046	0.852	0.148	14.780	**P<0.05**
Kováks et al.	0.865	0.130	0.005	0.930	0.070	0.028	P>0.05
Zakizadeh et al.	0.720	0.260	0.020	0.150	0.850	0.126	P>0.05
Pawar et al.	0.540	0.400	0.060	0.746	0.254	0.160	P>0.05
Balogh et al.	0.826	0.166	0.008	0.909	0.091	0.220	P>0.05
Hradecká et al.	0.914	0.086	0	0.957	0.043	0.632	**P<0.05**
Balogh et al.	0.803	0.186	0.011	0.896	0.104	0.021	P>0.05
Mohammadabadi et al.	0.544	0.311	0.146	0.699	0.301	7.051	**P<0.05**
Misrianti et al.	0.890	0.110	0	0.940	0.060	1.273	P>0.05
Heidari et al.	0.781	0.205	0.013	0.884	0.116	0.100	P>0.05
Akyuz et al.	0.770	0.060	0.170	0.840	0.160	100.326	**P<0.05**
Hadi et al.	0.390	0.610	0	0.690	0.310	28.981	**P<0.05**
Hartatik et al.[a]	0.840	0.160	0	0.920	0.080	0.140	P>0.05
Hartatik et al.[b]	0.790	0.210	0	0.900	0.100	0.587	P>0.05
Molee et al.	0	0.857	0.143	0.572	0.428	129.938	**P<0.05**
Ozdemir et al.	0.500	0.480	0.020	0.740	0.260	10.818	**P<0.05**
Sonmez et al.	0.410	0.490	0.100	0.652	0.348	0.618	P>0.05
Amiri et al.	0.778	0.185	0.037	0.870	0.130	12.783	**P<0.05**

Forest plots related to meta-analysis of genotypes and alleles is shown in *Fig. 1*. As a result of meta analysis, LL genotype pooled ratio of 0.710 (0.633-0.777) was calculated higher than LV and VV genotypes and L allele pooled ratio of 0.818 (0.751-0.871) was calculated higher than V allele *(Table 4)*.

Kendall's Tau-b correlation coefficient was calculated in the Begg and Mazumdar rank correlations statistics to determine publication bias. This coefficient is expected to be close to 1 and the p value is expected to be greater than 0.05. The results of Begg and Mazumdar Rank Correlations test revealed that there was no publication bias in the study samples for all genotypes and allele frequency ratios (P>0.05) *(Table 5)*. In the *Fig. 2*, funnel plots related to study sample of LL, LV and VV genotypes and L, V alleles are shown.

According to the genotype pooled ratios calculated as a result of meta analysis, the frequencies determined for all populations (n=4583) weren't found in the Hardy-Weinberg equilibrium in terms of bGH-AluI polymorphism. (χ^2=6.804, P=0.009) *(Table 6)*.

In the study, secondly, meta-analysis was performed with 6 studies in which the means of lactation milk yield were determined in the genotypes of bGH-AluI polymorphism, and the mean milk yields of the genotype subgroups were compared. High heterogeneity was determined between studies according to mean of milk yields (Q=60926.647, P<0.001, I^2=99.972). Therefore, random effect model was chosen in meta analysis and the overall mean of milk yield in the studies was found as 6.86 (5.83-7.89) L x1000. As a

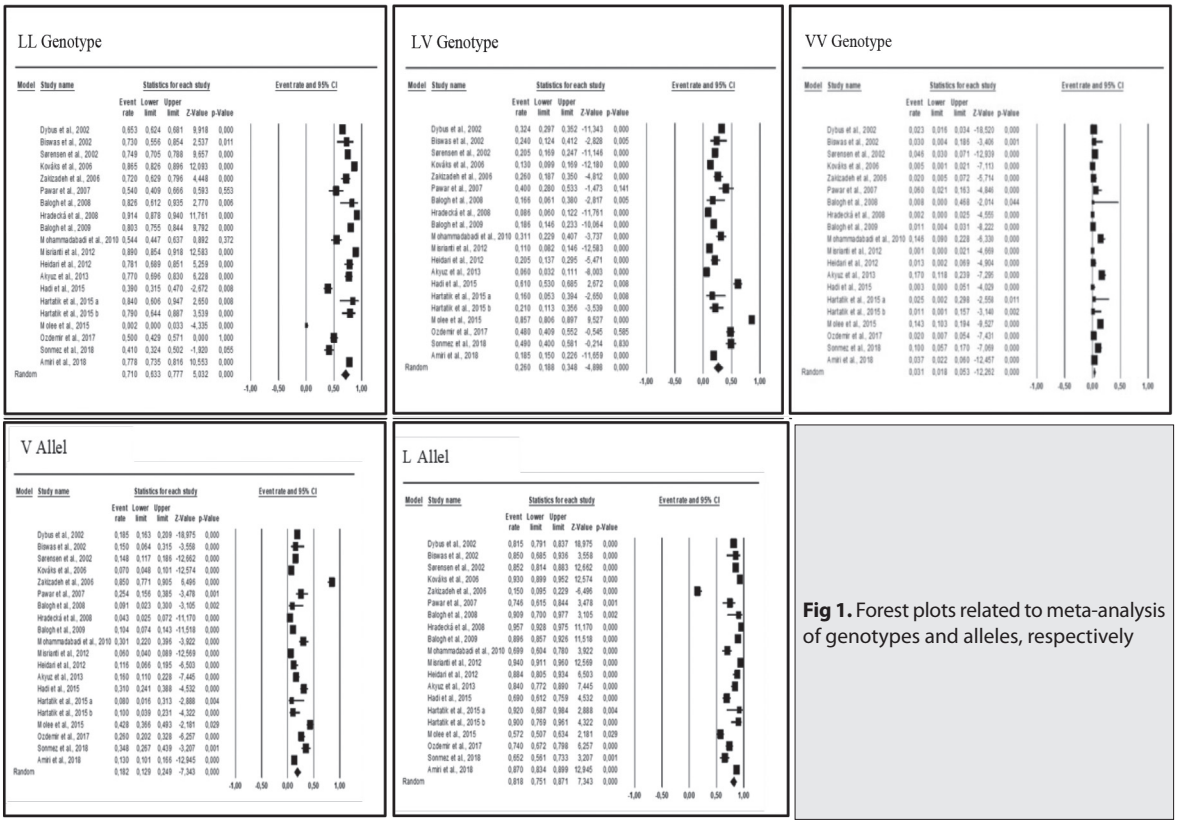

Fig 1. Forest plots related to meta-analysis of genotypes and alleles, respectively

Table 4. *Assessment of heterogeneity between studies for genotype and allele frequencies and calculated pooled ratios*

Variables		Genotype and Allele Frequency Ratios			Heterogeneity			
		Pooled Ratio	95% Confidence Interval	P Values	Cochran's Q	P Values	I^2 (%)	τ^2
Genotype	LL	0.710	0.633-0.777	<0.001	386.335	<0.001	95.082	0.545
	LV	0.260	0.188-0.348	<0.001	565.344	<0.001	96.639	0.837
	VV	0.030	0.018-0.053	<0.001	154.038	<0.001	87.665	1.034
Allel	L	0.818	0.751-0.871	<0.001	402.647	<0.001	95.281	0.743
	V	0.182	0.129-0.249	<0.001	402.647	<0.001	95.281	0.743

n: *Total number of cattle inclded in meta analysis,* ***Q:*** *Weighted sum of squares of observed effect sizes* ***I^2:*** *The proportion of true variance to observed variance,* ***τ^2:*** *Estimate of the between-study variance in true effects*

Table 5. *Publication bias tests of genotype and allele frequencies studies*

Variables		Begg and Mazumdar Rank Correlation Test	
		Kendall's Tau-b Correlation Coefficient	P Values
Genotype	LL	-0.068	0.673
	LV	-0.026	0.871
	VV	-0.100	0.538
Allel	L	0.079	0.627
	V	-0.079	0.627

result of the Begg and Mazumdar rank correlation test, it was observed that there was no publication bias in the study sample regarding mean of milk yields (P=0.325). The mean of milk yields for LL, LV and VV genotypes were calculated as 6.64, 6.96, 7.13 (x1000, Liter), respectively with the subgroup analysis. However, in subgroup analysis, there was no significant difference between genotypes for mean of milk yields (Q= 0.086, df= 2, P=0.958) *(Table 7)*. Forest plot of subgroup analysis of milk yield's (x1000, Liter) mean according to genotypes is shown in *Fig. 3*.

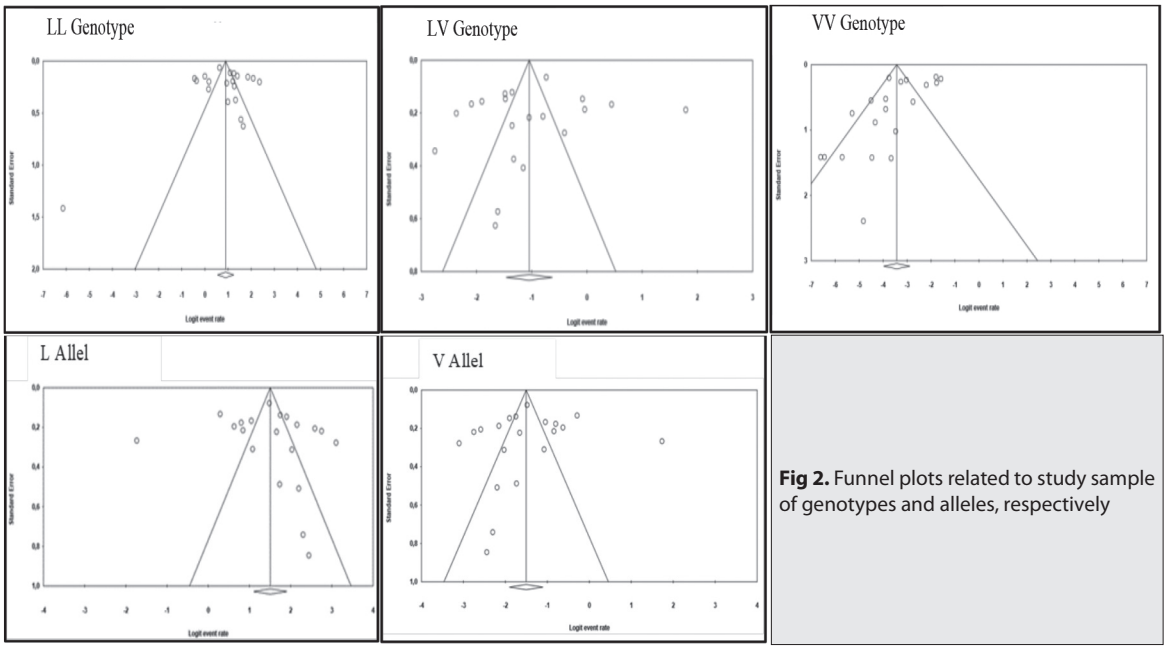

Fig 2. Funnel plots related to study sample of genotypes and alleles, respectively

Table 6. *Test of consistency with Hardy-Weinberg equilibrium in terms of bGH-AluI polymorphism according to the calculated genotype pooled ratios*

Statistics	Genotype			Statistical Significant
	LL	LV	VV	
Pooled Ratios (%)	0.710	0.260	0.030	χ^2=6.804 P=0.009 (df=1)
Calculated Frequencies (n)	3166	1256	161	

Table 7. *Subgroup analysis results and mean of milk yields (x1000, Liter) by genotypes*

Genotype	Number of Study	Mean of Milk Yields	95% Confidence Interval of Milk Yields
LL	7	6.64	4.50-8.78
LV	7	6.96	5.21-8.71
VV	4	7.13	4.32-9.94

Model	Group by Genotype	Study name	Statistics for each study						
			Mean	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
	LL	Kováks et al., 2006 a	10003,010	83,323	6942,653	9839,701	10166,319	120,052	0,000
	LL	Pawar et al., 2007 a	3156,000	16,614	276,033	3123,437	3188,563	189,957	0,000
	LL	Hadi et al., 2015 a	9317,000	70,562	4979,051	9178,700	9455,300	132,039	0,000
	LL	Hartatik et al., 2015 a 1	4000,000	390,000	152100,000	3235,614	4764,386	10,256	0,000
	LL	Hartatik et al., 2015 b 1	5660,000	313,842	98497,059	5044,880	6275,120	18,035	0,000
	LL	Ozdemir et al., 2017 a	7023,800	40,804	1664,970	6943,826	7103,774	172,135	0,000
	LL	Sonmez et al., 2018 a	7274,500	22,770	518,451	7229,873	7319,127	319,484	0,000
Random	LL		6641,275	1091,484	1191338,299	4502,005	8780,545	6,085	0,000
	LV	Kováks et al., 2006 b	10334,660	232,143	53890,222	9879,669	10789,651	44,519	0,000
	LV	Pawar et al., 2007 b	3895,000	16,203	262,545	3863,242	3926,758	240,384	0,000
	LV	Hadi et al., 2015 b	8979,000	54,005	2916,565	8873,152	9084,848	166,262	0,000
	LV	Hartatik et al., 2015 a 2	5190,000	132,791	17633,333	4929,735	5450,265	39,084	0,000
	LV	Hartatik et al., 2015 b 2	5790,000	496,667	246677,778	4816,551	6763,449	11,658	0,000
	LV	Ozdemir et al., 2017 b	7157,200	39,294	1544,028	7080,185	7234,215	182,144	0,000
	LV	Sonmez et al., 2018 b	7354,600	20,272	410,944	7314,868	7394,332	362,800	0,000
Random	LV		6960,369	890,472	792940,648	5215,076	8705,663	7,816	0,000
	VV	Kováks et al., 2006 c	10158,000	925,263	856112,295	8344,517	11971,483	10,978	0,000
	VV	Pawar et al., 2007 c	3803,000	31,177	972,000	3741,894	3864,106	121,981	0,000
	VV	Ozdemir et al., 2017 c	7007,700	331,100	109627,210	6358,756	7656,644	21,165	0,000
	VV	Sonmez et al., 2018 c	7836,900	78,635	6183,480	7682,778	7991,022	99,662	0,000
Random	VV		7128,383	1432,456	2051929,721	4320,822	9935,945	4,976	0,000
Random	Overall		6888,509	621,626	386418,667	5670,145	8106,873	11,081	0,000

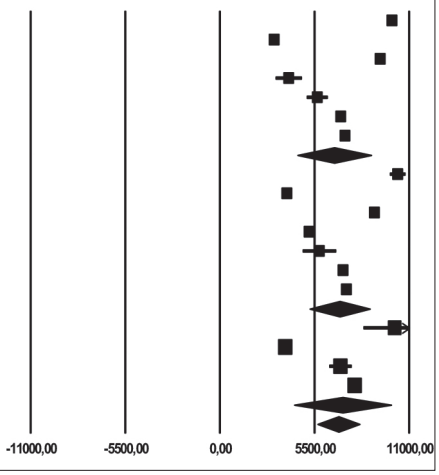

Fig 3. Forest plot of subgroup analysis of milk yields's (x1000, Liter) mean by genotypes

Suplementary Table. *References of studies included in meta analysis*	
No	**References**
1	**Dybus A:** Associations between Leu/Val polymorphism of growth hormone gene and milk production traits in Black-and-White cattle. *Arch. Tierz., 45* (5): 421-428, 2002.
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DISCUSSION

The genetic structure of the population is defined by the frequency of the genotypes present in the population and the frequency of the alleles of a gene, in terms of the feature under consideration [15]. It is necessary to study large samples in determining the genetic structure because when working with small samples, the probability of statistically significant differences between genotypes decreases. However, in cases where it is necessary to work with a small sample, the same hypothesis needs to be repeated at different times or by different researchers, and if the number of research exceeds 4, the results can be re-evaluated by meta-analysis. The results obtained by meta analysis can be used in the sample size calculation of new researches and comparisons can be made [16,17].

Since it has the potential to be a marker and is the most common polymorphism in the Holstein breed, in this study, genotype and allele frequencies and relations with milk yield characteristics obtained from studies investigating the bGH-*Alu*I polymorphism revealed by *Alu*I restriction enzyme in Holstein cattle were meta-analized. In terms of bGH-AluI polymorphism performed in Holstein cattle breeds, 4583 cattle in 20 studies on LL, LV and VV genotypes and L and V allell frequency ratios were included in the meta-

analysis. Since the high heterogeneity was determined between studies in all genotypes and allele frequencies, the DerSimonian-Laird method was used in calculating pooled ratios, because the random effect model was least affected by the outlier value.

According to the calculated pooled ratios, the LL genotype frequency ratio is highest (0.710); The VV genotype frequency ratio was the lowest (0.030). Thus, the L allele frequency pooled ratios (0.818) was calculated higher than the V allele frequency ratio (0.182). These results were similar to those of the studies included in the meta-analysis. The LL genotype frequency ratio was highest in most of the studies (17/20); included in the meta-analysis except three studies [18-20]. The VV genotype frequency ratio was reported to be higher than the LV genotype frequency ratio in one study [21] and the lowest in others (18/20). In addition, VV genotype could not be detected in five studies. It has been reported that the frequency ratio of the L allele is higher than the frequency ratio of the V allele in nineteen studies.

Hardy-Weinberg equilibrium is the basis of genetic inferences in the field of population genetics. According to the Hardy-Weinberg equilibrium, genotype frequencies do not change from generation to generation in populations that do not have selection, mutation, migration, and randomly mating. The Hardy-Weinberg equilibrium for samples randomly taken from a population is determined by the Pearson's $\chi 2$ compatibility test. In cases where the sample size is not sufficient, the prerequisites for suitability tests cannot be fulfilled and the reliability of the obtained results is low [22,23]. In this study, it was observed that 5 of the 20 studies included in the meta-analysis had been performed with a small sample size (<55). The bGH-AluI polymorphism genotype frequencies of the majority of studies (11/20) were found to be compatible with the Hardy-Weinberg equilibrium [23]. As the result of meta analysis, it was seen that all studies combined (n=4583) were in Hardy-Weinberg equilibrium those genotype frequencies were determined according to the calculated pooled ratios (P=0.009). The amount of heterozygosity determined in a population is the most important feature used in determining the genetic diversity of that population [15]. The variation in heterozygosity (LV) values observed in studies included in the meta-analysis (0.060-0.857) was found to be quite wide. This ratio was calculated as 0.260 as a result of meta analysis. Thus, closer results to the population parameter could be obtained with a large sample.

In previous studies, it has been reported that bGH-*Alu*I polymorphism in bGH gene, which is accepted as a potential candidate gene for genetic selection studies in Holstein cattle, has effects on meat and milk yield characteristics. In addition, studies have also been conducted in Holstein cattle to report that this polymorphism is associated with increased body weight [24,25]. Different results were obtained in the studies investigating the effect of bGH gene genotypes on milk yield characteristics in Holstein cattle. Many studies have reported that milk yield characteristics do not vary with respect to genotypes [18-20,26-28]. However, in some studies, cattle with LL genotypes were reported to have higher milk yield [4,29], whereas in two studies reported higher milk yield in cattle with LV genotype [5,30]. In the present study, it was determined by meta-analysis that the relationship between bGH-*Alu*I polymorphism genotypes and milk yield in different Holstein populations did not differ statistically. Meta-analysis is a statistical method used to systematically compile the results of scientific research carried out independently of each other and to combine these results with quantitative methods and present them as a summary of finding. This method has increased significantly and its use has become widespread day by day.

In this study, the relationship between genotype and allele frequencies and milk yield from the studies investigating bGH-*Alu*I polymorphism in Holstein cattle was synthesized by meta-analysis. Thus, closer results to the population parameter could be obtained in the phenotype-genotype relationships. It is thought that the results obtained in the present study will contribute to the development of new strategies required for rational production in dairy cattle.

However, within the scope of the study, more articles should be obtained in order to make the results more reliable as well as in order to compare a large number of groups on the result variability and to rank among the groups, the application of newly developed network meta-analysis methods is of importance for breeding. It is necessary to carefully select and analyze the studies to be included in the analysis in order to obtain correct results in applications and also to use the appropriate statistical model and to interpret the obtained analysis results correctly.

Conflict of Interest

The authors declare that there are no conflicts of interest

Statement of Author Contributions

Conception and design: A. Akçay, B. Akyüz Acquisition of data: A. Akçay, F. Daldaban, E. Çelik. Analysis and interpretation of data: A. Akçay, E. Çelik. Drafting the article: A. Akçay, K. Arslan, B. Akyüz. Revising it for intellectual content: A. Akçay, K. Arslan, B. Akyüz. Final approval of the completed article: A. Akçay, F. Daldaban, E. Çelik, K. Arslan, B. Akyüz

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Research Article

The Effect of Greater Celandine Active Ingredient Chelidonine on Isolated Rat Bladder and Trachea Smooth Muscles and Primary Lung and Kidney Cell Lines [1]

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Abstract

This study aims to explore the pharmacodynamics of chelidonine, the active ingredient in greater celandine *(Chelidonium majus* L.), on *in vitro* rat bladder and trachea tissue, and evaluate its cell protective effects on primary lung and kidney cell lines. The study was carried out via repeated applications of acetylcholine, atropine, verapamil and oxybutynin, alongside Ca^{++} in a calcium-free environment, on urinary bladder tissue, and repeated applications of acetylcholine, atropine, carbachol and mecamylamine on trachea tissue. At the same time, cell viability and catalase and superoxide dismutase activity was measured on primary cell lines obtained from lung and kidney tissue samples. The study has shown that chelidonine has a relaxant effect on bladder and trachea tissues, and it may be mentioned that this effect is produced via muscarinic receptors. In addition, chelidonin caused a statistically insignificant increase in cell viability in primary lung and kidney cell lines at increasing doses (1 and 4 µg/mL), but this increase remained at the control group level. In contrast, chelidonin caused a significant decrease in cell viability at the same cell lines at doses of 8 and 16 µg/mL. In conclusion, it is suggested that greater celandine, which is used in folk medicine, and its active ingredient chelidonine might have beneficial effects on asthma, urinary incontinence and other urinary tract and respiratory diseases among others.

Keywords: *Chelidonine, Urinary bladder, Trachea tissue, Smooth muscle, Cell culture, Rat*

Kırlangıç Otu Etkin Maddesi Chelidoninin İzole Rat İdrar Kesesi ve Trakea Düz Kasları ve Primer Akciğer ve Böbrek Hücre Hatları Üzerine Etkisi

Öz

Bu çalışmada, kırlangıç otu *(Chelidonium majus* L.) etkin maddesi chelidoninin *in vitro* rat idrar kesesi ve trakea dokusu üzerindeki farmakodinamik etkilerinin değerlendirilmesi ve primer akciğer ve böbrek hücre hatları üzerine hücre koruyucu etkilerinin araştırılması amaçlandı. Çalışma, idrar kesesi dokusunda tekrarlı yapılan asetilkolin, atropin, verapamil ve oksibutinin ile kalsiyumsuz ortamda Ca^{++} uygulamalarıyla, trakea dokusuna ise tekrarlı uygulamalarla asetilkolin, atropin, karbakol ve mekamilamin verilerek gerçekleştirildi. Aynı zamanda, alınan akciğer ve böbrek dokularından elde edilen primer hücre hatları üzerinde hücre canlılığı ölçümleri, katalaz ve süperoksit dismutaz enzim aktivitelerinin tayini yapıldı. Çalışma sonucunda, kırlangıç otu etkin maddesi chelidoninin idrar kesesi ve trakea dokuları üzerinde gevşetici etkisinin olduğu belirlendi ve chelidoninin bu etkisini muskarinik reseptörler üzerinden gösterdiğinden söz edilebilir. Ayrıca, chelidonin artan dozlarda (1 ve 4 µg/mL) primer akciğer ve böbrek hücre hatlarında hücre canlılığında istatistiksel olarak önemsiz bir artışa neden oldu, ancak bu artış kontrol grubu seviyesinde kaldı. Buna karşılık chelidonin aynı hücre hatlarında 8 ve 16 µg/mL dozlarında hücre canlılığında önemli bir düşüşe neden oldu. Sonuç olarak, halk arasında tıbbi amaçlarla kullanılan kırlangıç otunun ve etkin maddesi chelidonin astım, üriner inkontinans, idrar yolları ve solunum sistemi hastalıkları vb. durumlarda faydalı etkileri olabileceği görüşüne varıldı.

Anahtar sözcükler: *Chelidonin, İdrar kesesi, Trakea dokusu, Düz kas, Hücre kültürü, Rat*

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INTRODUCTION

Greater celandine *(Chelidonium majus* L.) is an herbaceous perennial plant from the Papaveraceae family, containing colored benzophenanthridine alkaloids. The plant has a long history of use in the treatment of various illnesses, particularly in European traditional medicine as well as Chinese herbal medicine [1-3]. In Turkey, *C. majus*, known with the names swallowwort and hilaliye, is a common ingredient in folk medicine [4]. The healing properties of the plant are mainly associated with the high content of biologically active compounds, and it is used in herbal medicine against bronchitis, jaundice, warts, skin cancer, digestive system conditions and eye diseases as well as for protection of the liver [1,3-5]. In particular, the dried flower-bearing branches, infusion, latex, tincture, leaf sap and decoction of *C. majus* L. are used for medicinal purposes. Greater celandine has sedative, hypnotic, analgesic, anti-inflammatory, anti-microbial, antitumoral, antiviral and laxative properties [2,6,7].

Chelidonium majus L. contains several types of alkaloids, such as the benzylisoquinoline group, which includes benzophenanthridines (chelidonine, chelerythrine, iso-chelidonine, sanguinarine), as well as protoberberines (berberine, coptisine, dihydrocoptisine, stylopine) and protopines (protopine). From these main alkaloids, chelidonine, coptisine and berberine are generally obtained from the top parts of the plant, while sanguinarine and chelerythrine are derived from the roots [2,5]. Chelidonine displays anti-inflammatory, immunomodulator, analgesic, antimicrobial, antiviral and antifungal properties [8,9].

The number of studies that explore the pharmacodynamics of chelidonine is limited [10-13]. While there are studies that focused at the plant extract alone, there are discrepancies in the results of studies that explore plant extracts and active ingredients, depending on the selection of animal species and tissue types used in the study. At the same time, researchers have voiced the need to supplement existing studies with more in-depth ones that investigate not only the active ingredients, but also their mechanisms of action. On the other hand, cell culture studies have shown that chelidonine has cytotoxic, apoptotic, antiproliferative, etc. effects on various cancer cell lines (e.g. human leukemia cells CEM, MT-4, MOLT-4 and Jurkat, hepatocarcinoma HepG2, breast cancer cells MCF-7, pancreatic and colon cancer cell lines) [5,6,9,14,15]. However, studies are mainly focused on the anticancer effects of chelidonine. This study aims to explore the pharmacodynamics of chelidonine, the active ingredient in greater celandine, on *in vitro* bladder and trachea tissue, and evaluate its cell protective effects on primary lung and kidney cell lines.

MATERIAL and METHODS

Drugs

The active ingredient used in the study, chelidonine (54274) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, US) and used to create a 1 mg/mL stock solution in ultrapure water. The remainder of the agents, namely acetylcholine (A6625), verapamil (V4629), oxybutynin (O2881), carbachol (C4382) and thiazolyl blue tetrazolium bromide (M5655) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, US); penicillin/streptomycin/amphotericin B (450-115 EL) from Wisent Inc. (Canada); atropine (226680100) from Acros Organics (Belgium); mecamylamine (M202600) from Toronto Research Chemicals Inc. (Ontario, Canada); and calcium chloride ($CaCl_2$, 328757) from Carlo Erba Reagents S.A.S. (Italy). All agents used in the study were at analytical grade.

Animals

The study used 36 adult Wistar Albino rats of 6 to 9 months of age, each weighing 250±20 g, which were raised at Sivas Cumhuriyet University Laboratory Animals Department. The study was conducted with Sivas Cumhuriyet University Animal Experiments Local Ethics Board (CÜHADYEK) approval 65202830-050.04.04-49 dated April 6, 2016.

Pharmacodynamics Study

The animals used in the study were anesthetized before being euthanized via cervical dislocation. Immediately afterwards, the urinary bladder and trachea tissues were removed. Urinary bladders were taken out through an incision made at the abdominal area, taking care to prevent damage to the samples. Each isolated bladder was placed in Krebs solution (in mM: NaCl 118; KCl 4.6; $NaHCO_3$ 25; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 10; EDTA 0.025; pH 7.4) before creating tissue preparations in the form of strips with dimensions of 2 mm x 0.5 mm x 10 mm. These preparations were placed in an isolated organ bath at a temperature of 37°C, continuous aeration using a gas mix of 95% O_2-5% CO_2, and inside 5 mL of krebs solution [16,17]. Tracheal tissues were removed without delay through an incision made at the neck area of the animals. Isolated trachea samples were placed in Krebs solution (in mM: NaCl 118; KCl 4.6; $NaHCO_3$ 25; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 10; pH 7.4). Afterwards, tracheal rings of 4 to 5 mm in length, fixed using stainless steel rings, were hanged in an isolated organ bath inside of krebs solution [18]. Isometric smooth muscle movements of urinary bladder and tracheal tissues inside the isolated organ baths were monitored and recorded using a "force transducer" (Force Displacement Transducer-FDT 05, Commat İletişim Ltd., Turkey) and an "acquisition system" (MP150 Biopac System, Commat İletişim Ltd., Turkey). The tissue samples were placed under 1.000 mg of tension and given at least 1 h to acclimatize to the environment, during which the krebs solution was replaced every 15 min. Once they reached equilibrium, the bladder and trachea tissues were stimulated using acetylcholine at EC_{50} values (10^{-6} M and 10^{-5} M, respectively). Following the contraction response, krebs solution in the baths were replaced; the samples

were washed and left to reach equilibrium once again. Afterwards, the following protocols were followed [17-20].

Single and cumulative dose chelidonine applications to urinary bladder smooth muscle and trachea tissue: Chelidonine was applied to the urinary bladder smooth muscle in single (10^{-7}; 10^{-6}; 10^{-5}; 10^{-4}; 10^{-3} M) and cumulative doses (10^{-7}-10^{-3} M, at 3 min intervals) to identify the dosage with the highest response (n=7). The same method was applied to tracheal tissues using the same dosages (n=8). As it was observed that cumulative dose applications to tracheal tissue produced more pronounced results, it was decided to focus further on cumulative dosages.

Acetylcholine application to urinary bladder smooth muscle and tracheal tissue after incubation in chelidonine: Chelidonine was applied to the bladder tissues in study dose and to tracheal tissues in cumulative doses, followed by 20 min of incubation. Afterwards, both urinary bladder and tracheal tissues were treated with a single dose of acetylcholine (10^{-6} M and 10^{-5} M, respectively) (n=8). Acetylcholine was also applied to urinary bladder smooth muscles and tracheal tissues in cumulative doses (10^{-8}-10^{-3} M) to determine its effects (n=6).

Chelidonine application to urinary bladder after incubation in atropine, verapamil and oxybutynin: Chelidonine was applied to the tissues in study dose followed by the application of acetylcholine (10^{-6} M), and the results were recorded (n=6). After the tissue samples are washed and left to reach equilibrium, they were incubated in antagonist atropine (10^{-6} M), verapamil (10^{-7} M) and oxybutynin (10^{-7} M), followed by an application of chelidonine in study dose.

Chelidonine application to trachea tissue after incubation in atropine, carbachol and mecamylamine: Trachea tissues were treated with chelidonine in cumulative-dose followed by the application of acetylcholine (10^{-5} M), and the results were recorded (n=6). Tracheal tissue samples were washed and left to reach equilibrium after replacement of the krebs solution, and then incubated in atropine (10^{-6} M), carbachol (10^{-6} M) and mecamylamine (10^{-5} M), followed by a cumulative-dose application of chelidonine.

1mM $CaCl_2$ application to urinary bladder smooth muscle in non-calcium Krebs solution after incubation in chelidonine and verapamil: The tissue samples were placed in a non-calcium krebs solution and treated with 1 mM $CaCl_2$, and the results were recorded (n=5). Afterwards, the samples were washed and left to reach equilibrium after replacement of the krebs solution. The tissue samples were treated with chelidonine in study dose and verapamil (10^{-7} M), and after 10 min of incubation, 1 mM $CaCl_2$ was added without washing the samples, and the results were recorded.

Cell Culture Study

Primary cell lines were used in this study. Primary cell lines were obtained from the lung and kidney tissues taken from the laboratory rats used in the study, in accordance with the method reported by Freshney [21]. The cells were put into flasks (10^6 cells/mL). After being prepared for chelidonine application, cell lines were left for 24 h to reach equilibrium. At the end of this period the samples were treated. Using the stock solution, five different concentrations of chelidonine were prepared (1, 2, 4, 8 and 16 µg/mL) and applied to the samples [14]. The control group was applied ultrapure water in equal volume. The control group and chelidonine-applied cells were left to incubate for 24 h. After incubation, cell viability and antioxidant enzyme (catalase-CAT and superoxide dismutase-SOD) activity measurements were taken. Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity analysis. In the MTT analysis, chelidonin (1-16 µg/mL) was applied to the cells planted in 96-well plates, with 10.000 cells per well, and after 24 h incubated, the plate was incubated with a mediocre 0.5 mg/mL MTT containing 5% CO_2 and 37°C for 4 h. At the end of the period, the MTT solution was removed and the cells were lized with dimethylsulfoxide (DMSO). The plate was then placed on an orbital shaker for 6 minutes and then absorbance at 570 nm was read using a microplate reader (spectrophotometer) [15]. In CAT and SOD enzyme activity assays, cells were planted in 24-well plates with 10^4 cells in each well and grown for 24 h, then concentrations of chelidonin 1-16 µg/mL were applied. The cells were incubated for 24 h and the CAT and SOD enzyme activity measurements were taken at the end of the period. The test kit for the measurement of CAT and SOD enzyme activities was used in line with the protocol recommended by the producer (Cayman Chemical Company, US).

Statistical Analysis

Results of the study were presented as mean and standard error of the mean (SEM). Contraction responses were expressed as apparent affinity constant (pD_2), with the pD_2 value was given as the negative logarithm of the molar agonist concentration that produces 50% of the maximum response produced by acetylcholine ($pD_2 = -\log EC_{50}$). Percentage of the corresponding maximal responses to the active ingredient were calculated as a percentage of the maximal response to acetylcholine (E_{max}). Mann-Whitney U test was utilized to assess the difference between the tissue samples. Statistical significance was set at $P<0.05$. pD_2, EC_{50}, E_{max} and IC_{50} values were calculated by interpolating the figures using GraphPad Prism (Version 8.2.0), while statistical analyses were made using SPSS (Version 23).

RESULTS

Pharmacodynamics Results

Single and cumulative dose chelidonine applications to urinary bladder smooth muscle and trachea tissue: It was observed that chelidonine has a relaxant effect

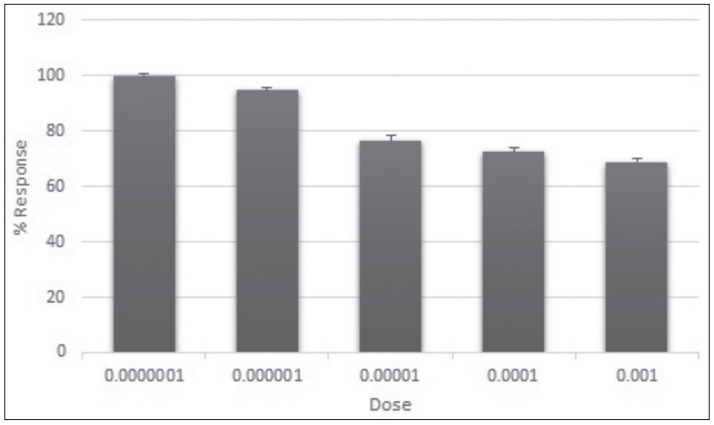
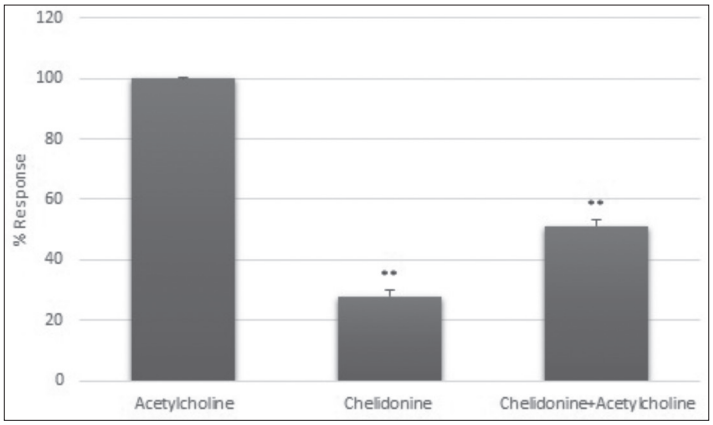


Fig 1. Single dose responses to chelidonine in urinary bladder

Fig 2. Relaxation responses of chelidonine and chelidonine + acetylcholine according to acetylcholine in the urinary bladder (** P<0.01)



on urinary bladder and trachea tissues, but the effect was not cumulative. In cumulative dose applications to urinary bladder and trachea tissues, pD_2 ($-logEC_{50}$) and E_{max} values were 5.024±0.24 and 101.5±2.74%; 4.870±0.01 and 100.7±0.09%, respectively. The chelidonine dose 10^{-5} M, which was shown to produce a better response based on the EC_{50} values obtained from single and cumulative dose applications to the urinary bladder, was selected for the study dose for the subsequent protocols. In single dose applications, % response for 10^{-5} M chelidonine dose was found to be 76.42±1.72% *(Fig. 1)*.

Acetylcholine application to urinary bladder smooth muscle and tracheal tissue after incubation in chelidonine: In urinary bladder tissues, 10^{-5} M dose of chelidonine induced a response of 27.96% by itself, and 51.16% when combined with acetylcholine (P<0.01) *(Fig. 2)*. Application of cumulative doses (10^{-7}-10^{-3} M) of chelidonine to trachea tissues followed by acetylcholine (10^{-5} M) resulted in an increase in contraction response; however, no correlation was observed between the dose and response. Application of cumulative doses of acetylcholine to bladder and trachea tissues provided pD_2 ($-logEC_{50}$) and E_{max} values of 3.718±0.24 and 120.0±16.29%; 3.672±0.52 and 119.8±15.79%, respectively.

Chelidonine application to urinary bladder after incubation in atropine, verapamil and oxybutynin: The contraction of the bladder on single-dose chelidonine (10^{-5} M) application (26.31%) showed a reduction in the presence of atropine (16.30%), verapamil (14.49%) and oxybutynin (10.92%) (P<0.01). Responses to acetylcholine (10^{-6} M) in the presence of the same antagonists showed a significant decline (13.25%, 74.40% and 37.13%, respectively), revealing a statistically significant difference from chelidonine responses (P<0.01) *(Fig. 3)*.

Chelidonine application to trachea tissue after incubation in atropine, carbachol and mecamylamine: It was noted that the presence of atropine, carbachol and mecamylamine affected the contraction of the trachea in response to cumulative doses of chelidonine (10^{-7}-10^{-3} M) (P>0.05). A statistically significant difference was observed between the responses to acetylcholine (10^{-6} M) in the presence of the same agents (14.54%, 62.66% and 40.39%, respectively) and chelidonine responses (P<0.01) *(Fig. 4)*.

1mM $CaCl_2$ application to urinary bladder smooth muscle in non-calcium Krebs solution after incubation in chelidonine and verapamil: The tension difference observed after incubation with 1 mM $CaCl_2$ in non-calcium krebs solution was 24.50 mg. Application of 1 mM $CaCl_2$ after single-dose chelidonine and verapamil incubation was shown to increase chelidonine response *(Fig. 5)*.

Cell Culture Results

MTT results: Cell viability was shown to be at a maximum in primary lung and kidney cells treated with 4 µg/mL

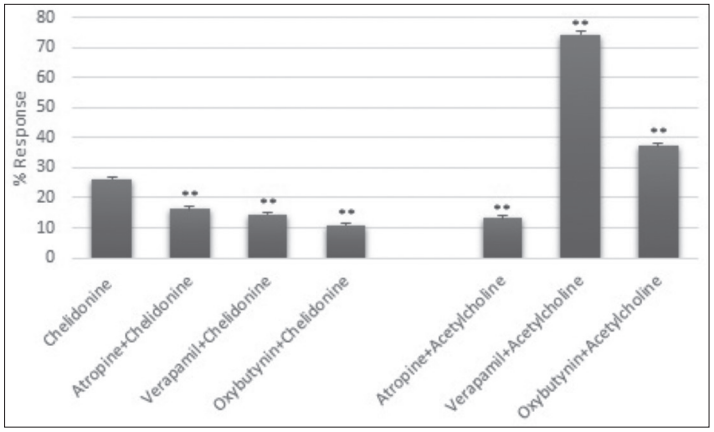
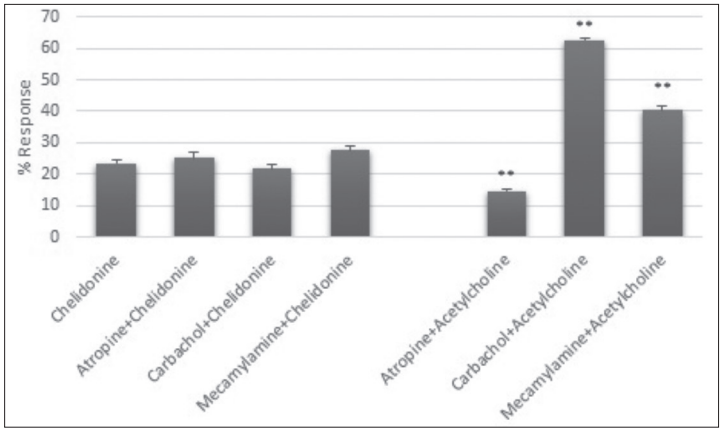


Fig 3. Antagonist+chelidonine and antagonist + acetylcholine relaxation responses in bladder (** P<0.01)

Fig 4. Agonist and antagonist chelidonine, agonist and antagonist acetylcholine relaxation responses in trachea tissue (** P<0.01)



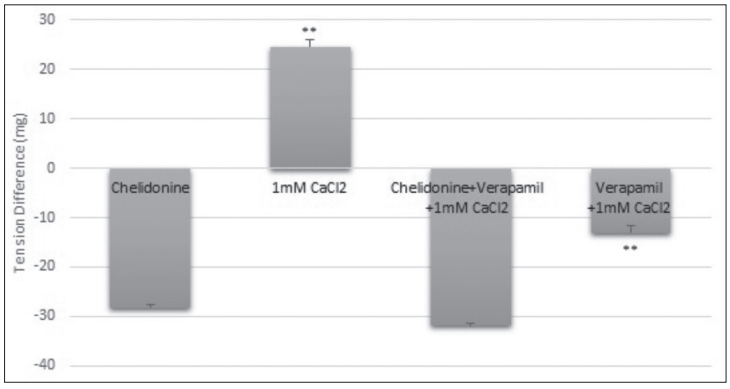


Fig 5. 1 mM $CaCl_2$ relaxation responses in bladder after chelidonine and verapamil (10^{-7} M) incubation in non-calcium krebs solution (** P<0.01)

of chelidonine, with further increases in concentration reducing cell viability *(Fig. 6)*. Chelidonine application revealed an IC_{50} value of 10.91 µg/mL in primary lung cells, and 12.19 µg/mL in primary kidney cells.

CAT and SOD results: Application of chelidonine in different concentrations (1-16 µg/mL) to primary lung and kidney cells revealed a concentration-based change in CAT and SOD levels in comparison to the control group. Application of chelidonine in a concentration of 4 µg/mL to primary lung cells (P<0.05) and in concentrations of 2 and 4 µg/mL to primary kidney cells (P<0.05) showed a statistically significant increase in CAT levels compared to control *(Fig. 7)*. Likewise, SOD levels displayed a statistically significant change from control group results when chelidonine was applied to primary lung cells in a concentration of 4 µg/mL (P<0.05) and to primary kidney cells in concentrations of 1, 2 and 4 µg/mL (P<0.05, P<0.01, P<0.05) *(Fig. 8)*.

DISCUSSION

The relationship between plants and humans is as old as history itself, and experiences passed down from generation to generation have resulted in a wealth of knowledge and treatment methods in time. It is necessary to identify the chemical composition and active ingredients, as well as their mechanisms of action, of these plants and herbs that are used in traditional medicine, in order to ensure proper, efficient and sufficient use [7,22].

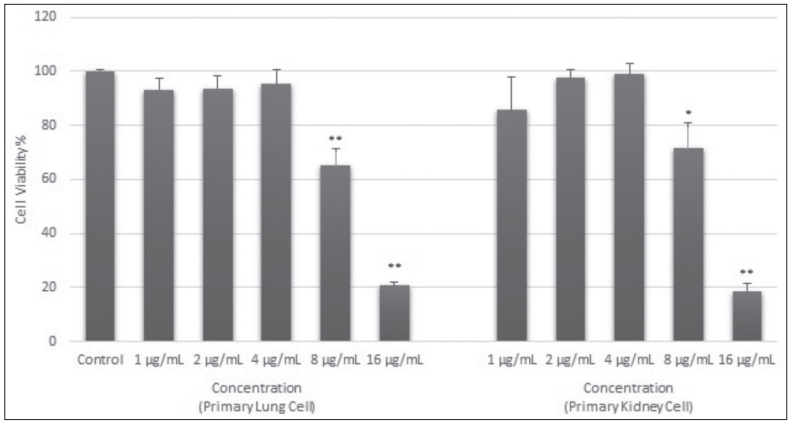
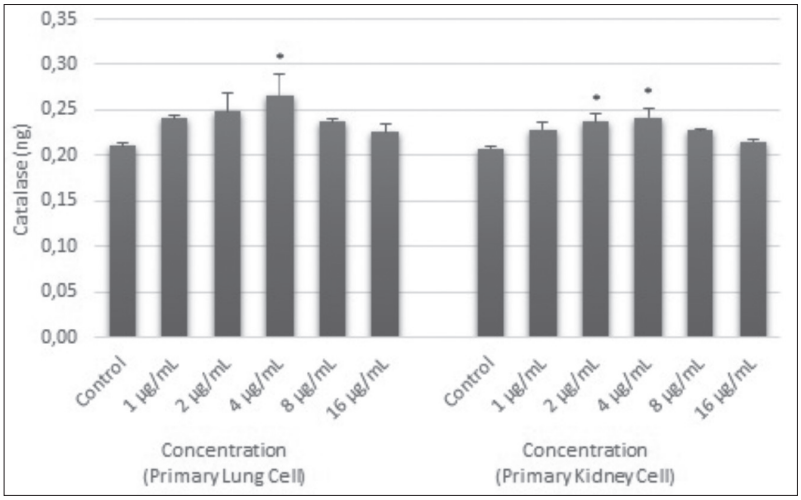


Fig 6. Cell viability % values of primary lung and kidney cells treated with chelidonine (* P<0.05; ** P<0.01)

Fig 7. Catalase values of primary lung and kidney cells treated with chelidonine (* P<0.05)



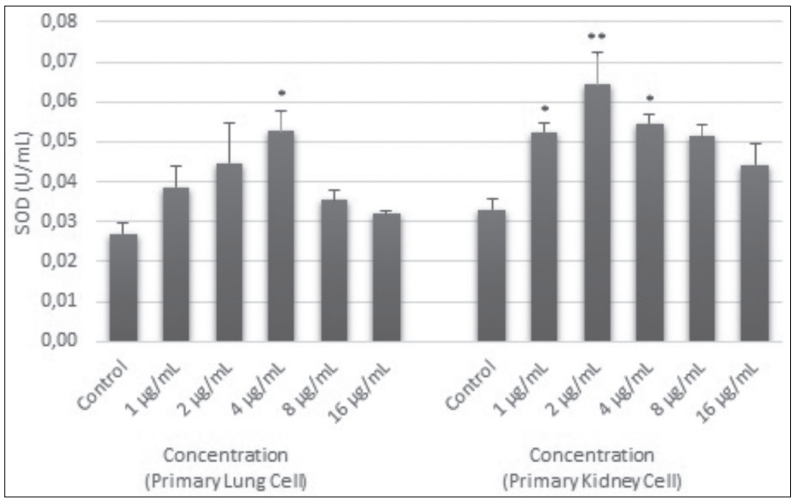


Fig 8. SOD values of primary lung and kidney cells treated with chelidonine (* P<0.05; ** P<0.01)

This study aimed to assess the pharmacodynamics of chelidonine, the active ingredient in greater celandine, a plant used in folk medicine against a variety of conditions, on *in vitro* rat bladder and trachea tissue, and to evaluate its cell protective effects on primary lung and kidney cell lines. The results of this study have shown that chelidonine has a relaxant effect on urinary bladder and trachea tissues *(Fig. 1)*. In a study by Hiller et al.[11], which explored the effects of the primary alkaloids derived from the ethanolic extract of greater celandine *(C. majus* L.), namely chelidonine, protopine and coptisine on guinea pig ileum tissue, it was shown that chelidonine had a similar effect to papaverine on ileum tissue when the tissue is stimulated with $BaCl_2$, and that carbachol had an antagonistic relationship with chelidonine. Chelidonine was also reported to be a musculotropic agent with a spasmolytic effect similar to papaverine [13], with possible antioxidant properties as a spasmolytic [12], as well as a

relative antagonistic relationship with acetylcholine [10]. This study has found that chelidonine responses showed a decrease compared to acetylcholine responses, and that acetylcholine applied together with chelidonine resulted in a comparatively smaller % increase in responses *(Fig. 2)*. Chelidonine is suggested to have an antagonistic effect with acetylcholine, which is consistent with the findings of previous studies. In a study that explored the effect of raw *C. majus* and *Corydalis lutea* extracts on isolated rat ileum tissues induced with acetylcholine, the contraction produced by acetylcholine was found to be antagonistic (12.7%) with *C. majus* extract applied in a dose of $2.0x10^{-4}$ g/mL. As a result, the study revealed that *C. majus* has an antispasmodic effect [10] On the other hand, studies show that the urinary bladder smooth muscle of various species (including humans) contains all muscarinic receptor subtypes, but the M2 and M3 receptors are dominant. However, it is emphasized that muscarinic M3 receptors are primarily responsible for contraction of the bladder smooth muscle [23,24]. While atropine has the same antagonistic effect on all subtypes of muscarinic receptors [24], oxybutinine has an antagonistic effect on the M3 receptor subtype with a higher affinity than other muscarinic subtypes [25]. Verapamil is a kind of calcium channel blocker [26]. In this study, it was observed that the relaxing effect of chelidonin in the urinary bladder was strengthened in the presence of atropine, verapamil and oxybutin ($P<0.01$) *(Fig. 3)*. In addition, the M2 and M3 subtypes of muscarinic receptors mediate effects in airway smooth muscles, and the M3 receptor plays an important role in contraction of airway smooth muscles [24]. At the same time, acetylcholine, which is synthesized in non-neuronal cells such as epithelial cells of the airways, also plays a role in the regulation of basic cell functions [27]. Carbachol, a cholinergic agonist substance, is more associated with the activation of muscarinic receptors (M3) [28], while mecamilamine is a potent ganglion blocker [29]. In this study, the contraction in tracheal tissue in response to cumulative doses of chelidonine (10^{-7}-10^{-3} M) is observed to show significant changes in the presence of carbacol *(Fig. 4)*, leading to the conclusion that chelidonine and carbachol have an antagonistic relationship, which is consistent with previous studies. This study also suggests that chelidonine is attracted to cholinergic muscarinic receptors; however, it reduces the effect of agonists due to its lower potency compared to acetylcholine. In addition, application of 1mM $CaCl_2$ to bladder tissue in non-calcium krebs solution after chelidonine and verapamil incubation was found to enhance chelidonine response *(Fig. 5)*, revealing the important role of the calcium ion in acetylcholine responses.

As with many potent natural products or mainly secondary plant metabolites, the active ingredient of greater celandine, chelidonine has been subject to several studies in the context of treatment of various cancer types. The cytotoxic effect mechanisms of greater celandine alkaloids show considerable variety, particularly in terms of their potential contribution to various cell death signal pathways. Previous studies showed remarkable differences in the DNA binding and sub-contribution capacity of benzophenanthridines isolated from this plant, with alkaloids such as sanguinarine and chelerythrine displaying high DNA binding capacity, while that of chelidonine was negligible [6,30]. On the other hand, it is associated with oxidative stress in lipid peroxidation, which results in the production of reactive oxygen species (ROS), affects the cell membrane and induces tissue damage, cell membrane deterioration and disruption. SOD and CAT are regarded as sensitive bio-indicators of oxidative stress, and are known to protect against ROS [31]. This study has shown that application of chelidonine in a concentration of 4 µg/mL to primary lung cells ($P<0.05$) and in concentrations of 2 and 4 µg/mL to primary kidney cells ($P<0.05$) resulted in statistically significant increase in CAT levels compared to the control group *(Fig. 7);* likewise, concentrations of 4 µg/mL in primary lung cells ($P<0.05$) and 1, 2 and 4 µg/mL in primary kidney cells ($P<0.05$, $P<0.01$, $P<0.05$) increased SOD levels significantly compared to control *(Fig. 8)*.

A study by Kaminskyy et al.[32] assessed the apoptotic, cytotoxic and DNA-damaging effects of isoquinoline alkaloids sanguinarine, chelerythrine and chelidonine on primary rat spleen cells and rat lymphocytic leukemia cell lines (L1210). According to the results of the study, chelidonine does not exhibit a clear cytotoxic or DNA-damaging effect on either cell types; however, it was observed to completely block cell growth in L1210 cells, preventing L1210 proliferation. In another study, chelidonine was assessed for its potential cytotoxic effect on pancreas, colon and breast cancer cell lines, primary endometrial cancer cells and murine pancreatic adenocarcinoma cells [5]. This study revealed that an application of 4 µg/mL of chelidonine to primary lung and kidney cells increased cell viability to its maximum (95.27% and 98.88%, respectively) *(Fig. 6)*. However, further increases in concentration reduced cell viability and increased cytotoxic effect.

In this study, it is found that chelidonine, the active ingredient in greater celandine, has a relaxant effect on urinary bladder and trachea tissues and that it is attracted to the cholinergic muscarinic receptors; however, it is also observed that the agent reduces the effect of agonists due to its lower potency compared to acetylcholine, and that calcium ions also play an important role in acetylcholine responses. As suggested before, chelidonine is a musculotropic agent with a spasmolytic effect similar to papaverine that acts through multiple mechanisms, and it may be mentioned that chelidonine acts through muscarinic receptors. The results of this study revealed that chelidonine has an effect on cholinergic receptors in the presence of agonists and antagonists. In addition, chelidonine caused a statistically insignificant increase in cell viability in primary lung and kidney cell lines at increasing doses (1 and 4 µg/mL), but this increase remained at the

control group level (P>0.05) *(Fig. 6)*. In contrast, chelidonine caused a significant decrease in cell viability at the same cell lines at doses of 8 µg/mL (respectively; P<0.01, P<0.05) and 16 µg/mL (P<0.01). In conclusion, it is suggested that the medicinal plant greater celandine and its active ingredient chelidonine might have beneficial effects on asthma, urinary incontinence and other urinary tract and respiratory diseases among others.

Conflict of Interest

The authors declared that there is no conflict of interest.

Statement of Author Contributions

E. Arslanbaş conceived the ideas of the study and writing manuscript; H. O. Doğan, H. Güngör, M. O. Atasoy and A. S. Kumru performed data collection and laboratory analysis; E. Arslanbaş, H. Kara and N. H. Turgut performed data analysis.

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Short Communication

Differentiation of *Staphylococcus pseudintermedius* in the *Staphylococcus intermedius* Group (SIG) by Conventional and Molecular Methods

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Abstract

Staphylococcus pseudintermedius is a member of the *Staphylococcus intermedius* group (SIG) comprising representatives with similar phenotypic characteristics. The purpose of this study was to optimise a method for accurate differentiation of *S. pseudintermedius* in clinical samples from dogs and cats based on PCR-RFLP analysis, following the key biochemical tests for SIG group assignment, and to determine the prevalence of *S. pseudintermedius* compared to other members of the SIG group. To implement this, 158 isolates from dogs and 17 from cats, 4 reference strains - *S. intermedius* ATCC 29663, *S. pseudintermedius* LMG 22219, *Staphylococcus delphini* ATCC 49171, *Staphylococcus aureus* ATCC 25922 and 5 field strains of *S. aureus* were analysed. The generated PCR-RFLP profile *(pta* gene, *MboI* restriction enzyme) clearly differentiated *S. pseudintermedius* within the SIG group by having two distinctive bands of 213 and 107 bp, respectively. The data showed a predominance of *S. pseudintermedius* in clinical samples from dogs, while no *S. pseudintermedius* were found among the cat's samples. In conclusion, due to the atypical biochemical reactions found in some *S. pseudintermedius* isolates (14.6%), precise identification should be based on an extensive phenotypic assay together with a molecular biological method or the latter alone.

Keywords: *Staphylococcus pseudintermedius, Biochemical markers, PCR-RFLP analysis, differentiation, Staphylococcus intermedius Group*

Staphylococcus intermedius Grubundaki (SIG) *Staphylococcus pseudintermedius*'un Geleneksel ve Moleküler Yöntemlerle Ayırdedilmesi

Öz

Staphylococcus pseudintermedius, benzer fenotipik özelliklere sahip temsilciler içeren *Staphylococcus intermedius* grubunun (SIG) bir üyesidir. Bu çalışmanın amacı, köpek ve kedilerden elde edilen klinik örneklerde SIG grubu ataması için temel biyokimyasal testleri takiben PCR-RFLP analizine dayanarak *S. pseudintermedius*'un doğru şekilde ayırdedilmesi için bir yöntemi optimize etmek ve SIG grubunun diğer üyelerine kıyasla *S. pseudintermedius*'un yaygınlığını belirlemekti. Bu amaçla, köpeklerden elde edilen 158 ve kedilerden elde edilen 17 izolat, 4 referans suş - *S. intermedius* ATCC 29663, *S. pseudintermedius* LMG 22219, *Staphylococcus delphini* ATCC 49171, *Staphylococcus aureus* ATCC 25922 ve *S. aureus*'un 5 saha suşu analiz edildi. Oluşturulan PCR-RFLP profili *(pta* geni, *MboI* kısıtlama enzimi), *S. pseudintermedius*'u sahip olduğu 213 ve 107 bp'lik iki farklı bantla SIG grubu içinde belirgin şekilde ayırdetti. Veriler, köpeklerden alınan klinik örneklerde *S. pseudintermedius*'un baskın olduğunu gösterirken, kedi örneklerinde *S. pseudintermedius* belirlenmedi. Sonuç olarak, bazı *S. pseudintermedius* izolatlarında bulunan atipik biyokimyasal reaksiyonlar (%14.6) nedeniyle kesin tanı, kapsamlı bir fenotipik test ile birlikte kullanılacak moleküler biyolojik bir yöntem veya sadece moleküler yönteme dayanmalıdır.

Anahtar sözcükler: *Staphylococcus pseudintermedius, Biyokimyasal markerler, PCR-RFLP analizi, Ayırdetme, Staphylococcus intermedius Grubu*

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INTRODUCTION

Staphylococcus spp. are Gram-positive bacteria causing a variety of opportunistic infections in humans, domestic and wild animals [1]. Usually, the pathogenicity of these bacteria is associated with the production of coagulase enzyme, on the basis of which they are defined as coagulase-positive and coagulase-negative, respectively. The following coagulase-positive/variable species are essential for veterinary medicine -*Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus schleiferi* subsp. *coagulans*, *Staphylococcus hyicus*, *Staphylococcus lutrae*, *Staphylococcus delphini* and *Staphylococcus pseudintermedius* [2,3], while coagulase-negative ones are considered significant when isolated from sterile body sites or in community-acquired infections [4].

Until recently, *S. intermedius* was thought to be commensal on the skin and mucous membranes of various animal species such as dogs, cats, pigeons, minks, horses, foxes, raccoons, goats and gray squirrels [5]. According to Wang et al.[6] *S. intermedius* is the predominant bacterial agent responsible for the etiology of the superficial pyoderma and soft tissue infections in dogs. With the development of advanced molecular methods, in particular DNA sequencing technologies, a group of researchers have clarified that the isolates identified as *S. intermedius* by phenotype methods actually a compound of three species - *S. intermedius*, *S. pseudintermedius* and *S. delphini*, which form together the *S. intermedius* group (SIG) [2]. Subsequently, based on DNA-DNA hybridization techniques and multilocus sequence typing (MLST), it was found out that the main bacterium responsible for the etiology of skin infections in dogs, cats and humans was practically *S. pseudintermedius*, and *S. intermedius* colonised mainly pigeons. Thus, the earlier scientific publications describing *S. intermedius* are now regarded as reports on *S. pseudintermedius* [7-9]. In addition, the clinical significance of *S. delphini* isolated from different animal species is considered greater than previously thought [10,11].

The misidentification of the SIG group representatives is due to the high degree of similarity in their phenotypic characteristics, as well as the existence of different biotypes of *S. intermedius* showing 50 to 65% DNA homology, which can be different species such as *S. delphini* and *S. pseudintermedius* [8]. On the other hand, genetic methods for differentiating microbial species based on the 16S rRNA gene are useless in these staphylococci because of the established gene similarity of over 99% [2,12]. The literature overview reveals several genetic approaches for the identification of *S. pseudintermedius* such as - PCR-RFLP analysis, nuc-PCR, sodA /hsp60 sequencing, and MALDI-TOF-MS analysis [3,8,13,14].

The purpose of this study was to optimise a method for accurate identification of *S. pseudintermedius* in clinical samples from dogs and cats based on the standard polymerase chain reaction with subsequent restriction of amplified fragments - PCR-RFLP analysis, following the key biochemical tests for SIG group assignment, and to determine the prevalence of *S. pseudintermedius* compared to other members of the SIG group.

MATERIAL and METHODS

Bacterial Strains

One hundred and seventy five isolates relevant to the SIG group - from dogs (n=158) and cats (n=17), 4 reference strains as controls - *S. intermedius* ATCC 29663, *S. pseudintermedius* LMG 22219, *S. delphini* ATCC 49171, *S. aureus* ATCC 25922, as well as 5 field *S. aureus* strains of cow, sheep, chicken, dog and rabbit origin were included in the study. Clinical specimens from dogs included ear discharge (n=48), skin lesions (n=42), ocular discharge (n=29), wound discharge (n=17), urine (n=8), material of oral tongue and gingival lesions (n=11) and synovial fluid (n=3). Cat isolates originated from skin lesions (n=9), urine (n=5), and ear discharge (n=3). Samples were cultured on trypticase soy agar (Fluka, India) supplemented with 5% defibrinated sheep blood and on MacConkey agar (NCIPD, Bulgaria; HiMedia, India). The plates were incubated under aerobic conditions at 37°C for 24-48 h. The initial identification of significant staphylococcal species was based on Gram's staining, production of catalase and cytochrome oxidase enzymes, colonial characteristics and pigmentation, presence of complete haemolysis and the tube coagulase test with lyophilised rabbit plasma (NCIPD, Bulgaria).

Biochemical Profile

The biochemical profile of the isolates was determined on the basis of the following key tests: acetoin production in liquid medium with glucose, detection of β-galactosidase (4 mg disks, HiMedia, India), sensitivity to polymyxin B (300 U/disk, Oxoid, UK), utilisation of mannitol on mannitol-salt agar ((NCIPD , Bulgaria), of trehalose and maltose in liquid media (MkB Test, Slovak republic).

The tests were carried out in accordance with the manufacturer's instructions and the general rules for aseptic work in the microbiology laboratory [15].

Additionally, reference strains from the SIG group as well as selected clinical isolates were analysed with a semi-automatic BioLog phenotype identification system (BioLog, USA) with GenIII microplates according to the manufacturer's instructions. Briefly, wells were loaded with 100 µL of bacterial suspensions at a density of 90-98% determined on a turbidimeter (BioLog, USA) and the plates incubated at 33°C for 20-24 h.

PCR-RFLP Analysis

DNA was isolated using a QIAamp commercial DNA mini kit (Quiagen, GmbH, Germany) following the Gram-positive bacteria protocol requiring pre-treatment of the bacterial

stock with buffer containing 20 mg/mL lysozyme, 20 mM Tris HCl with pH 8.0, 2 mM EDTA and 1.2% Triton. DNA quality was determined spectrophotometrically at A260/A280 on a Gene Quant 1300 apparatus (Healthcare Bio-Sciences AB, Sweeden). The extracts were stored at -20°C until the start of the experiments.

PCR was performed with the primers targeting amplification of a 320 bp fragment of the phosphoacetyl transferase *(pta)* gene. The sequences of primers as follows: pta_f1, AAA GAC AAA CTT TCA GGT AA and pta_r1, GCA TAA ACA AGC ATT GTA CCG [13]. The PCR reaction adjusted in a final volume of 20 µL was as follows: primers 0.25 µM, 1 x PCR buffer, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, Taq polymerase 0.5 U/rxn (Canvax, Spain) and DNA 1 µL. The thermal conditions were adjusted in a gradient PCR machine included: initial denaturation at 95°C for 2 min followed by 30 cycles consisting of denaturation at 95°C for 60 sec, annealing at 53°C for 60 sec, elongation at 72°C for 60 sec and final amplification for 7 min at 72°C. Subsequently, 10 µL of amplified product were subjected to restriction with 1 µL of *MboI* (10 U/µL) according to the manufacturer's protocol (Canvax, Spain). The restricted products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide.

RESULTS

Bacterial isolates determined as suspicious for *S. pseudintermedius* based on their characteristics in the conventional microbiological tests until PCR-RFLP analysis was performed are presented in *Table 1*. The table shows that most (85.44%) of the isolates exhibit a typical *S. pseudintermedius* profile expressed in the presence of β-haemolysis, a positive test for proving free plasma coagulase within 2-4 h, negative test for acetoin production and β-galactosidase positivity (ONPG test), sensitivity to polymyxin B, acidification of mannitol, trehalose and maltose. Atypical reactions were found in 23 (14.6%) strains with respect to the presence of hemolysins and acetoin production in the VP test. These isolates were respectively γ-haemolytic (2.5%) and VP positive (12.0%). *Table 1* also shows the biochemical characteristics of the reference strains included in the study. The coagulase-positive isolates of cats exhibited a variability in VP and ONPG assays and were interpreted as resistant to polymyxin B, which was not in line with the profile for *S. pseudintermedius*.

The *S. intermedius* ATCC 29663, *S. delphini* ATCC 49171 and *S. pseudintermedius* LMG 22219 reference strains, and the two clinical isolates tested were successfully identified with the BioLog commercial system. The substrates used as carbon sources by the reference strains of the SIG group and two of the isolates identified as *S. pseudintermedius* with *MboI* restriction analysis are shown in *Table 2*. It is obvious that 14 of the substrates are completely utilised from the five strains tested. Only six substrates - stachyose, D-aspartic acid, D-serine, p-hydroxy-phenylacetic acid, D-malic acid and γ-amino-butryric acid, were not metabolised by the species tested, whereas no metabolic activity was recorded against D-cellobiose, D-raffinose, D-melibiose, D-salicin, 3 methyl glucose, L-fucose, L-rhamnose, bromo-succinic acid and β-hydroxy-D, L-butyric acid with exception of one of the strains showing borderline activity. The profile of *S. pseudintermedius* strains differs only in their inability to use seven substrates as a carbon source, unlike *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 and, with respect to four substrates, borderline activity or lack thereof in clinical isolates was recorded.

The results of the PCR-RFLP analysis are presented on *Fig. 1*. The restriction profile of the dog strains suspected to be *S. pseudintermedius* consisted of two bands at 213 bp and 107 bp, respectively. *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 did not exhibit the *MboI* restriction site, whereas the tested *S. aureus* strains showed a restriction profile with a band of 160 bp. Amongst the cat isolates, the *MboI* restriction profile specific for *S. pseudintermedius* was not established.

DISCUSSION

In routine bacteriological examinations, pathogenic staphylococci are frequently identified to the level of coagulase activity and no further identification is undertaken. On the other hand, the incorrect differentiation of

Table 1. *Biochemical profile of the isolates from dogs suspected to be S. pseudintermedius and the reference strains from the SIG group determined on the basis of key assays*

Bacterial Isolates and Reference Strains	Hemolysis	Coagulase	Acetoin	β-galactosidase	Polymyxin B	Mannitol	Trehalose	Maltose
S. pseudintermedius n=98	β	+	-	+	S	+	+	+
S. pseudintermedius n=19	β	+	+*	+	S	+	+	+
S. pseudintermedius n=4	ϒ*	+	-	+	S	+	+	+
S. pseudintermedius n=37	β	+	-	+	S	-	+	+
S. pseudintermedius LMG 22219	β	+	-	+	S	-	+	+
S. intermedius ATCC 29663	β	+	+	+	S	+	+	+
S. delphini ATCC 49171	β	+	-	-	S	+	+	+

*+ = positive reaction/test (including weakly positive and delayed positive reaction); - = negative reaction/test; S/R = sensitivity/resistance; * atypical reactions*

Table 2. *Biochemical profile data of S. intermedius ATCC 29663, S. delphini ATCC 49171, S. pseudintermedius LMG 22219, and two S. pseudintermedius clinical isolates (#1 and # 2) determined on the basis of 71 carbon sources in the BioLog GenIII plate*

Substrates	ATCC 29663	ATCC 49171	LMG 22219	#1	#2	Substrates	ATCC 29663	ATCC 49171	LMG 22219	#1	#2
Dextrin	+	+	B	B	B	Glycyl-L-Proline	+	+	+	-	B
D-Maltose	+	+	+	B	B	L-Alanine	+	+	+	+	+
D-Trehalose	+	-	+	+	+	L-Arginine	B	+	-	-	-
D-Cellobiose	B	-	-	-	-	L-Aspartic Acid	B	+	B	-	-
Gentiobiose	+	-	-	-	-	L-Glutamic Acid	+	+	+	B	B
Sucrose	+	+	+	+	+	L-Histidine	+	+	+	B	B
D-Turanose	+	+	+	-	+	L-Pyroglutamic Acid	B	+	-	-	-
Stachyose	-	-	-	-	-	L-Serine	+	+	+	+	+
D-Raffinose	B	-	-	-	-	Pectin	+	+	B	B	B
α-D-Lactose	+	+	+	+	+	D-Galacturonic Acid	+	+	B	B	-
D-Melibiose	B	-	-	-	-	L-Galactonic Acid Lactone	+	+	B	-	-
B-Methyl-D-Glucoside	+	-	+	+	+	D-Gluconic Acid	+	+	+	+	+
D-Salicin	B	-	-	-	-	D-Glucuronic Acid	-	+	-	B	-
N-Acetyl-D-Glucosamine	+	+	+	B	B	Glucuronamide	-	+	-	B	B
N-Acetyl-β-D-Mannosamine	+	+	-	-	-	Mucic Acid	B	+	-	-	-
N-Acetyl-D-Galactosamine	-	+	-	B	-	Quinic Acid	B	+	-	-	-
N-Acetyl Neuraminic Acid	+	+	+	+	+	D-Saccharic Acid	B	+	B	-	-
α-D-Glucose	+	+	+	+	+	p-Hydroxy-Phenylacetic Acid	-	-	-	-	-
D-Mannose	+	+	+	B	B	Methyl Pyruvate	+	B	B	+	B
D-Fructose	+	+	+	+	+	D-Lactic Acid Methyl Ester	-	B	B	-	-
D-Galactose	+	+	+	+	+	L-Lactic Acid	+	+	+	+	+
3 Methyl Glucose	B	-	-	-	-	Citric Acid	-	+	B	-	-
D-Fucose	+	-	-	-	-	α-Keto-Glutaric Acid	+	+	+	B	B
L-Fucose	B	-	-	-	-	D-Malic Acid	-	-	-	-	-
L-Rhamnose	B	-	-	-	-	L-Malic Acid	B	+	+	B	B
Inosine	+	B	+	B	+	Bromo-Succinic Acid	-	-	B	-	-
D-Sorbitol	-	+	B	-	-	Tween 40	+	+	-	-	-
D-Mannitol	+	+	+	+	+	γ-Amino-Butryric Acid	-	-	-	-	-
D-Arabitol	+	-	-	-	-	α-Hydroxy-Butyric Acid	+	+	+	B	B
Myo-Inositol	B	+	-	-	-	β-Hydroxy-D, L-Butyric Acid	-	B	-	-	-
Glycerol	+	+	+	+	+	α-Keto-Butyric Acid	+	+	+	B	B
D-Glucose-6-PO4	+	+	+	+	+	Acetoacetic Acid	+	+	B	B	B
D-Fructose-6-PO4	+	+	+	+	+	Propionic Acid	+	+	+	-	-
D-Aspartic Acid	-	-	-	-	-	Acetic Acid	+	+	+	B	B
D-Serine	-	-	-	-	-	Formic Acid	-	+	+	B	B
Gelatin	-	+	B	-	-						

B*=borderline activity*

coagulase-positive staphylococci is quite possible, especially for the representatives of the SIG group, due to their similar biochemical properties. The precise determination of these staphylococci is important for clinical practice with regard to the choice of antimicrobial agent associated with the correct interpretation of the minimum inhibitory

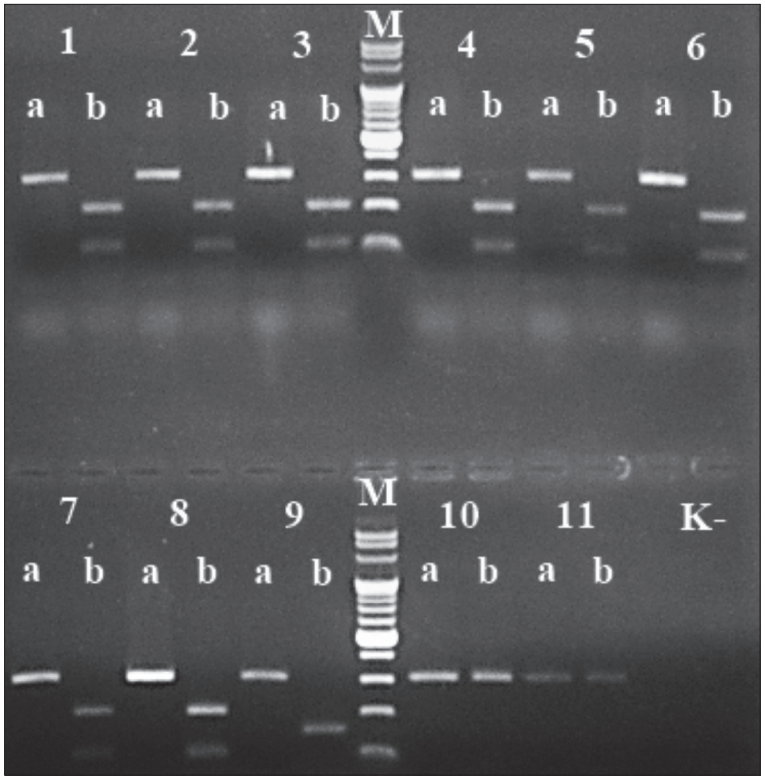

Fig 1. MboI restriction profile of *pta* PCR products in 2% agarose gel stained with ethidium bromide. Legend: 1-7 - *S. pseudintermedius* isolates; 8 - *S. pseudintermedius* LMG 22219; 9 - *S. aureus* ATCC 25922; 10 - *S. intermedius* ATCC 29663; 11 - *S. delphini* ATCC 49171; a - *pta* PCR product (320 bp); b - *pta* PCR-MboI product; M - molecular marker, 100 bp; K- negative control

concentrations and the resulting outcome of therapy [1,8,16], and because of the established zoonotic potential of *S. pseudintermedius* and *S. intermedius* [11].

In the present study, the isolates with a biochemical profile suspected for *S. pseudintermedius* were selected based on key biochemical markers for primary differentiation of clinically relevant staphylococci in veterinary medicine [15]. Key markers for the differentiation of the three staphylococcal species included in the test panel were the VP test and the ONPG test, which clearly categorised the reference strains. However, atypical reactions were found in *S. pseudintermedius* isolates relative to the production of acetoin in which *S. pseudintermedius* and *S. intermedius* profiles did not differ. The BioLog GenIII phenotypic identification system identified reference strains *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 with the highest probability of 1.000, whereas *S. pseudintermedius* LMG 22219 and both clinical isolates with a probability of 0.555 - 0.569, confirming the great biotype diversity of this bacterial species [8]. In this regard, we disagree with Devriese [2] who claims that there is no commercial kit available to identify *S. intermedius* and *S. delphini*. In addition, the updated BioLog system software includes the metabolic profile of *S. pseudintermedius*, which enables accurate identification at the phenotypic level. The carbon sources that did not detect metabolic activity in the reference strain and the clinical isolates compared to S. *intermedius* ATCC 29663 and *S. delphini* ATCC 49171 were N-acetyl-β-D-mannosamine, myo-inositol, L-arginine, L-pyroglutamic acid, mucic acid, quinic acid and Tween 40. Borderline activity or lack thereof in one of the clinical isolates was found against pectin, D-galacturonic acid, L-galactonic acid lactone and acetoacetic acid. With respect to the other biochemical reactions, detailed comparisons with other data published on *S. pseudintermedius* cannot be made due to the different substrates incorporated in the commercially used identification systems - BioLog in our study, API 50CH and API STAPH (BioMerieux), and STAPH-ZYM (Rosco) in the research of Devriese [2].

The PCR-RFLP method on which accurate identification of *S. pseudintermedius* isolates was based in this study was originally developed by Bannoehr et al.[13], and has been used successfully in other countries [17,18]. The method was also reproduced in our laboratory after optimisation of temperature parameters for hybridisation of primers at amplification of pta-PCR. The most distinct amplification products of the *pta* gene were visualised at 54°C, and the optimum denaturation, annealing and elongation time for each cycle was determined to be 45 s. Subsequent *MboI* analysis of PCR products of 320 bp clearly differentiates *S. pseudintermedius* isolates having a restriction site resulting in two bands of 213 and 107 bp, respectively. The restriction profile of *S. aureus* ATCC 25922 as well as the five *S. aureus* field strains differed by one visible band of about 160 bp in the agarose gel, which actually consists of two closely spaced bands - 156 and 164 bp, which are not established in the routine electrophoresis [13]. *S. intermedius* ATCC 29663 and *S. delphini* ATCC 49171 are not differentiated by this method as they do not have a restriction site and *MboI* products have not been realised.

The data in our study showed a predominance of *S.*

pseudintermedius in clinical samples from dogs. The species affiliation of the isolates found to be suspicious for *S. pseudintermedius* by conventional methods was confirmed by PCR-RFLP analysis. Only two skin lesion isolates showed no restriction site and could not be determined by the RFLP method. The involvement of *S. aureus* in infectious pathology in dogs in a previous study of ours was estimated at 16.1% [19]. All these data confirm the prevalence of *S. pseudintermedius* in dog specimens. With respect to cat isolates, *S. pseudintermedius* was not detected in any of the samples tested, which is consistent with the data of Bannoehr et al.[13], who found *S. pseudintermedius* in only one of the 14 cat isolates tested. Due to the limited number of feline isolates analysed, definitive conclusions regarding the prevalence of *S. pseudintermedius* in this animal species in our country cannot be made. Obviously, its prevalence ranges from 3.08% in healthy cats to 11.97% in sick cats, values found in Poland in a large animal study conhort [20]. The other isolates did not show a restriction profile and their precise determination will be the subject of future studies based on other modern approaches.

In conclusion, the results of this study demonstrate the usefulness of key biochemical tests to target the SIG group members, but the precise differentiation of *S. pseudintermedius*, *S. intermedius* and *S. delphini* should be based on extensive phenotypic analysis together with molecular biological methods, or the latter alone.

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Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

N.R. designed, carried out the laboratory work, and wrote the paper; S.K., A.A., A.R - contributed in collecting clinical samples; S.S. assisted in molecular analysis and final revision of the paper.

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Short Communication

Rickettsia aeschlimannii and *Wolbachia endosymbiont* in *Ctenocephalides canis* from Eurasian lynx *(Lynx lynx)* Near the China-Kazakhstan Border

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Abstract

Twenty-five dog fleas *(Ctenocephalides canis)* and five ticks identified as *Hyalomma asiaticum* were collected from a Eurasian lynx *(Lynx lynx)* pup in northwestern China. Molecular analyses of four genetic markers showed the presence of *Rickettsia aeschlimannii* DNA in 5 out of 20 fleas. Only rickettsial *17-kDa* gene was detected in the blood sample of the lynx. In addition, 2 out of 20 fleas were positive to *Wolbachia endosymbiont* by targeting *16S rDNA* while there was no *Bartonella* DNA found both in 5 ticks and 20 fleas by using *gltA* and *16S-23S ITS*. Our findings suggest that i) *C. canis* parasitizing wild Eurasian lynx harbors *R. aeschlimannii* and *Wolbachia endosymbiont* in the China-Kazakhstan border, and ii) *Wolbachia endosymbiont* in present study is closer to that in *C. canis* infesting sheltered dogs in Turkey.

Keywords: *Eurasian lynx, Ctenocephalides canis, Hyalomma asiaticum, Rickettsia aeschlimannii, Wolbachia endosymbiont*

Çin-Kazakistan Sınır Bölgesinde Bir Avrasya Vaşağındaki *(Lynx lynx) Ctenocephalides canis*'te Saptanan *Rickettsia aeschlimannii* ve *Wolbachia endosymbiont*

Öz

Çin'in kuzeybatısında bir Avrasya vaşağı *(Lynx lynx)* yavrusundan yirmi beş adet köpek piresi *(Ctenocephalides canis)* ve *Hyalomma asiaticum* olarak tanımlanan beş adet kene toplandı. Dört genetik markerin moleküler analizi, 20 pirenin 5'inde *Rickettsia aeschlimannii* DNA'sının varlığını gösterdi. Vaşağın kan örneğinde ise sadece riketsiyal *17-kDa* geni tespit edildi. Buna ek olarak, 20 pire'den 2'si *Wolbachia endosymbiont*'un hedef *16S rDNA*'sı yönünden pozitifken, *gltA* ve *16S-23S ITS* kullanılarak yapılan analizde 5 kenede ve 20 pirede *Bartonella* DNA'sına rastlanmadı. Bulgularımız, i) Çin-Kazakistan sınırında vahşi Avrasya vaşaklarını enfekte eden *C. canis*'in *R. aeschlimannii* ve *Wolbachia endosymbiont*'a konakçılık yaptığını ve ii) mevcut çalışmadaki Wolbachia endosymbiont'unun, Türkiye'deki barınak köpeklerini enfekte eden *C. canis*'teki ile daha yakın olduğunu göstermektedir.

Anahtar sözcükler: *Avrasya vaşağı, Ctenocephalides canis, Hyalomma asiaticum, Rickettsia aeschlimannii, Wolbachia endosymbiont*

INTRODUCTION

The Eurasian lynx *(Lynx lynx)* is a medium-sized wild cat (Mammalia: Carnivora: Felidae), designated as a Class II protected species in China [1]. This species is widely distributed in Europe, Central- and Northern Asia, including

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Kazakhstan, China and Korea [2]. *Rhipicephalus pusillus*, *Rhipicephalus turanicus*, *Rhipicephalus sp.*, *Ixodes ricinus*, *Ixodes ventalloi*, *Ctenocephalides canis*, *Pulex irritans*, *Spilopsyllus cuniculi* and *Ctenocephalides felis* were reported from Iberian lynx *(Lynx pardinus)* [3,4]. In North America, *Amblyomma americanum* and *Dermacentor variabilis* ticks were collected from the bobcat *(Lynx rufus)* [5,6]. When ticks infesting these two lynx species were molecularly screened for tick-borne pathogens, four *Rickettsia* species belonging to the spotted fever group (SFG), i.e. *Rickettsia helvetica*, *R. massiliae, R. monacensis* and *R. rickettsii*, were detected [6,7].

Rickettsia aeschlimannii, member of spotted fever group (SFG) rickettsiae, caused human infection [8], and was molecualrly detected in the blood of camels in Israel [9]. It was firstly found in *Rhipicephalus turanicus* ticks in 2015 in northwest China [10]. Wolbachia can inhibit the transmission of certain viruses and the infectivity of the malaria-causing protozoan, Plasmodium and filarial nematodes. Furthermore, Wolbachia can cause a form of conditional sterility that can be used to suppress populations of mosquitoes and additional medically important insects [11]. In this study, the ectoparasites of Eurasian lynx *(Lynx lynx)* was reported, and *Wolbachia* and *Rickettsia* were molecularly detected in arthropod vectors.

MATERIAL and METHODS

Twenty-five fleas and five ticks were collected from a road-killed Eurasian lynx pup, in Toli County (805 m above sea level; 82°41'30E 45°19'04N) in northwestern China in August, 2019. A blood sample was also collected from the Eurasian lynx pup. The sampled fleas and ticks were carefully surface-sterilized, washed in 3% hydrogen peroxide (H_2O_2) followed by 70% ethanol (EtOH) [12]. Genomic DNA was extracted individually from 20 fleas (the other five fleas were used as morphological identification by making slides [13]), 5 ticks and the pup's blood sample by using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The molecular identification was carried out by amplifying the mitochondrial *18S rRNA* and the cytochrome c oxidase subunit I *(cox1)* genes for fleas and the *16S rRNA* gene for ticks. PCR primers were shown in *Table 1*. The presence of rickettsial DNA was investigated by PCR amplification and sequencing of parts of the following four genetic markers: 17 kilodalton antigen *(17-kDa)*, citrate synthase *(gltA)*, cell surface antigen 1 *(sca1)*, and outer membrane proteins A *(ompA)* as described previously [14]. Phylogenetic relationships of the detected rickettsial agent were investigated by the Maximum Likelihood and Neighbor-Joining analyses. In addition,

Table 1. *Characteristics of amplified fragments and corresponding primer sequences*

Species	Gene		Primer	Fragment	Ref.
Tick	*16S rRNA*		F-CTGCTCAATGATTTTTTAAATTGCTGTGG	460	[14] [16]
			R-CCGGTCTGAACTCAGATCAAGT		
Flea	*18S rRNA*		F-CCTGAGAAACGGCTACCACATC	1150	
			R-GCATCACAGACCTGTTATTGC		
	cox1		F-GGTCAACAAATCATAAAGATATTGG	710	
			R-TAAACTTCAGGGTGACCAAAAAATCA		
Rickettsia	*17-kDa*	out	F-GCTTTACAAAATTCTAAAAACCATATA	434	[14]
			R-TGTCTATCAATTCACAACTTGCCGTT		
		in	F-GCTCTTGCAACTTCTATGTT		
			R-CATTGTTCGTCAGGTTGGCG		
	gltA	out	F-ATGACCAATGAAAATAATAAT	1178	[14]
			R-ATTGCAAAAAGTACAGTGAACA		
		in	F-GGAATCTTGCGGCATCGAGGATATG	931	
			R-CCATAGCTTTATAGATAATACCCG		
	sca1	out	F-GGTGATGAAGAAGAGTCTC	657	[14]
			R-CTCTTTAAAATTATGTTCTAC		
		in	F-GAGGTTTGTGGATGCGTGGT	553	
			R-ACTGTGACTTTAGTACCGACA		
	ompA	out	F-ATGGCGAATATTTCTCCAAAA	532	[14]
			R-AGTGCAGCATTCGCTCCCCCT		
		in	F-CTTAAAGCCGCTTTATTCACCACCTC	433	
			R-CCTGTATAATTATCGGCAGGAGC		
Wolbachia	*16S rDNA*		F-TTGTAGCCTGCTATGGTATAACT	936	[17]
			R-GAATAGGTATGATTTTCATGT		

Wolbachia and *Bartonella* were also molecularly detected by targeting *16S rDNA*, *gltA* and *16S-23S rRNA* intergenic spacer region, respectively [15].

RESULTS

The fleas were identified morphologically as *C. canis* according to the following key features *(Fig.1)*. The first spine is half as long as second spine of the genal comb. The pattern of metatibial formula of chaetotaxy is 2-2-2-2-2-1-1-3. The shape of the manubrium of the clasper expanded apically [18]. The sampled five ticks (3 male and 2 female) were identified as *Hyalomma asiaticum* by morphological observation *(Fig. 2)*. Briefly, Coxa I deeply was incised with two contiguous and unequal spurs. Legs harbor areas of enamelling. Cervical grooves were deep and long while lateral grooves short [19]. Then the morphological results were confirmed by amplifying *18S rRNA* (GenBank: MN565695), *cox1* (MN565696) and *16S rRNA* (MN625446) genes. Rickettsial DNA was detected in 5 out of 20 (25%) *C. canis* fleas, but not positive in any of the 5 *H. asiaticum* ticks *(Fig. 3-A)*. Interestingly, only the *17-kDa* gene was detected in lynx blood sample.

BLAST search results showed that sequences of three genes *(17-kDa*, *ompA*, and *sca1)* from the fleas were 100% identical to that of *R. aeschlimannii* isolate VGD7 from *C. felis* infesting domestic animals in Peru, while the obtained *gltA* sequence showed only 97.52% (908/931 bp) identity in the same context. All new sequences were deposited in GenBank *(17-kDa*: MN557354; *sca1*: MN557355; *gltA*: MN603746; *ompA*: MN557357). The phylogenetic analysis of concatenated sequences confirmed that the rickettsial agent in this study clustered closest to *R. aeschlimannii (Fig. 4)*.

Wolbachia DNA was also detected in 2 out of 20 (10%) *C. canis* fleas but negative in 5 ticks while there was no *Bartonella* DNA both in ticks and fleas *(Fig. 3-B)*. The phylogenetic analysis of *Wolbachia 16S rDNA* indicated that *Wolbachia endosymbiont* in present study was clustered into that in *C. canis* collected from sheltered dogs in Turkey *(Fig. 5)* [20].

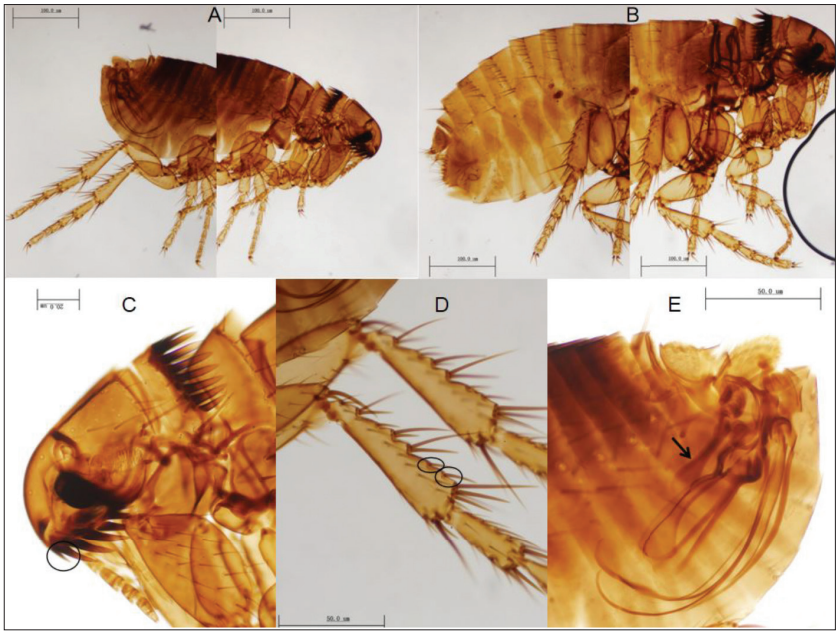

Fig 1. The key features of *C. canis* fleas. A: The male. B: the Female. C: The first and second spines of the genal comb. D: The metatibial formula of chaetotaxy. E: The shape of the manubrium of the clasper

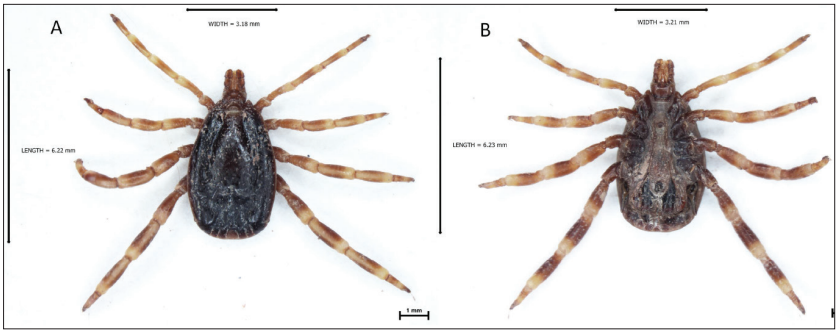

Fig 2. *Hyalomma asiaticum* collected from Eurasian lynx pup. A: Dorsal view. B: Ventral view

DISCUSSION

Hyalomma asiaticum is a highly abundant tick species in Xinjiang Uygur Autonomous Region (XUAR), Northwestern China. The main habitat of this tick species includes desert or semi-desert areas, where it is typically found on camel, sheep, cattle, horses and other livestock, but can also infest humans and wild animals [21]. In addition, *C. canis* (Siphonaptera: Pulicidae) is known as an ectoparasite of dogs and cats [13], Here *H. asiaticum* and *C. canis* are reported from Eurasian lynx.

Rickettsia aeschlimannii was previously detected in *Hyalomma aegyptium*, *H. turanicum*, *H. excavatum*, *H. impeltatum*, *H. dromedarii*, *Hyalomma marginatum* and *Hyalomma rufipes* ticks in Algeria, Israel, Southern Algeria, Morocco, Croatia, Spain, southern France, Portugal, Italy, Russia, Cyprus, Germany, Turkey, Hungary, and the Greek island of Cephalonia [9,16,22-24].

In 2015, *R. aeschlimannii* was detected in *Rhipicephalus turanicus* ticks from sheep in Yining County, XUAR [25]. Additional SFG rickettsiae shown to be present in other arthropods, such as *R. raoultii* and *R. slovaca* in the sheep ked, *Melophagus ovinus* (Diptera: Hippoboscidae) [16], as well

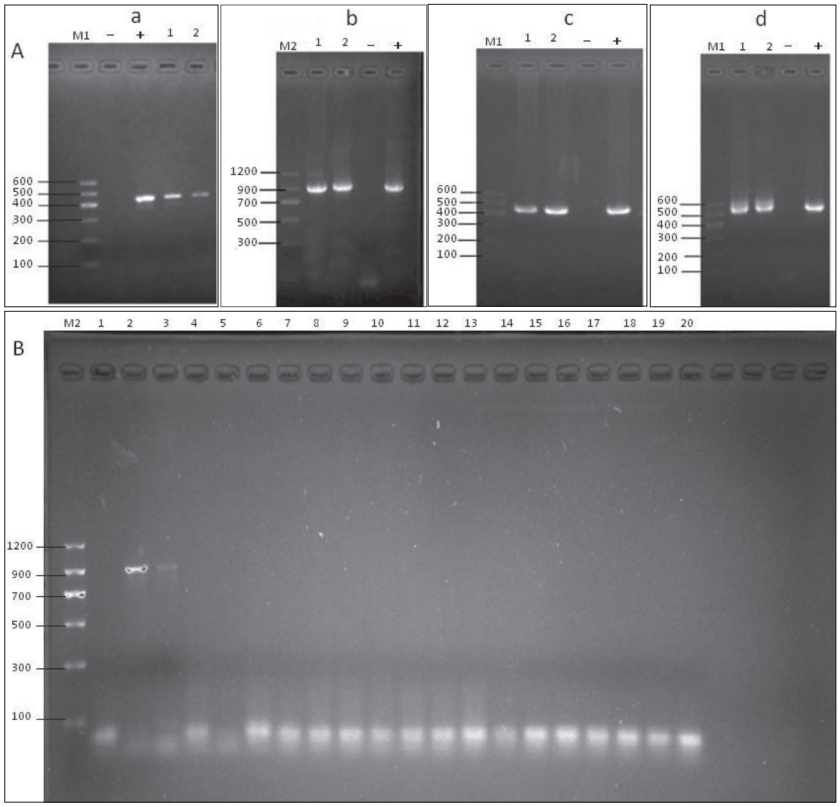

Fig 3. The PCR products of the Rickettsiae *17-KDa*, *gltA*, *ompA*, *sca1* genes and *Wolbachia 16S rDNA* genes. A: a, *17-KDa*. b, *gltA*. c, *ompA*. d, *sca1*. B: *16S rDNA*

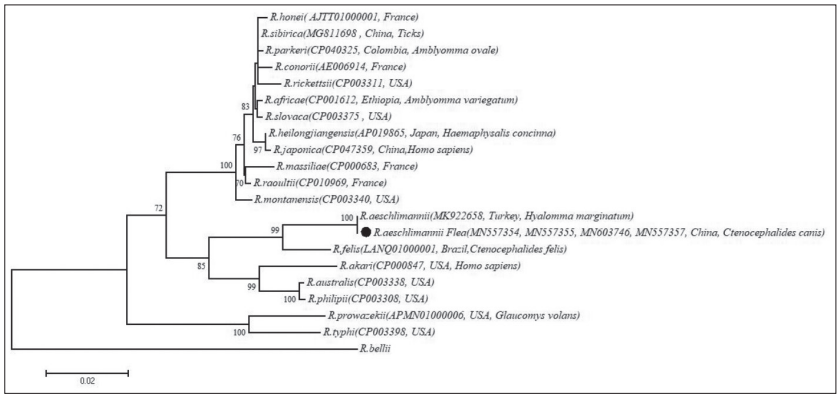

Fig 4. Phylogenetic tree of the *17-kDa-ompA-gltA-sca1* concatenated sequence of rickettsial agents in Eurasian lynx and infesting fleas. The new sequences provided by the present study are indicated by a black circle (containing the accession number). The phylogenic analyses were conducted using MEGA 6.0 software

as Candidatus *R. barbariae* in the flea *Vermipsylla alakurt* [26]. Concerning the flea species, *C. canis*, it is known to carry *R. felis*, as reported in Uruguay [27]. Here *R. aeschlimannii* and *Wolbachia endosymbiont* were detected in *C. canis* from Eurasian lynx. Interestingly, the rickettsial *17-kDa* gene was also detected here from lynx blood (MN603747). These findings suggest that *C. canis* parasitizing wild Eurasian lynx harbors *R. aeschlimannii* and *Wolbachia endosymbiont* in the China-Kazakhstan border.

In previous studies, *C. canis* was collected from feral cat *(Felis catus)*, feral dog *(Canis lupus)*, red fox *(Vulpes vulpes)* and chilla foxes *(Pseudalopex griseus Gray)* [10,28]. In this study, *C. canis* was sampled from Eurasian lynx and *Wolbachia* DNA was also detected in *C. canis*.

In conclusion, the flea species *C. canis* and the tick species *H. asiaticum* were reported in Eurasian lynx *(Lynx lynx)*. Furthermore, *R. aeschlimannii*, an emerging member of the spotted fever group (SFG) [25], was molecularly detected in *C. canis* fleas. These findings extend our knowledge on the geographical distribution of *R. aeschlimannii* and the range of its carriers, which now includes not only ticks but also fleas. In addition, the presence of *Wolbachia endosymbiont* in *C. canis* might be more related to flea species rather than infesting hosts.

Acknowledgments

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Author's Contributions

Gang Liu, Shuo Zhao, Xinli Gu and Yuanzhi Wang conceived and designed the study, and wrote the manuscript. Gang Liu, Shuo Zhao, Meihua Yang and Wurelihazi Hazihan performed the experiments, and analyzed the data. Sándor Hornok critically revised the manuscript. All authors read and approved the final manuscript.

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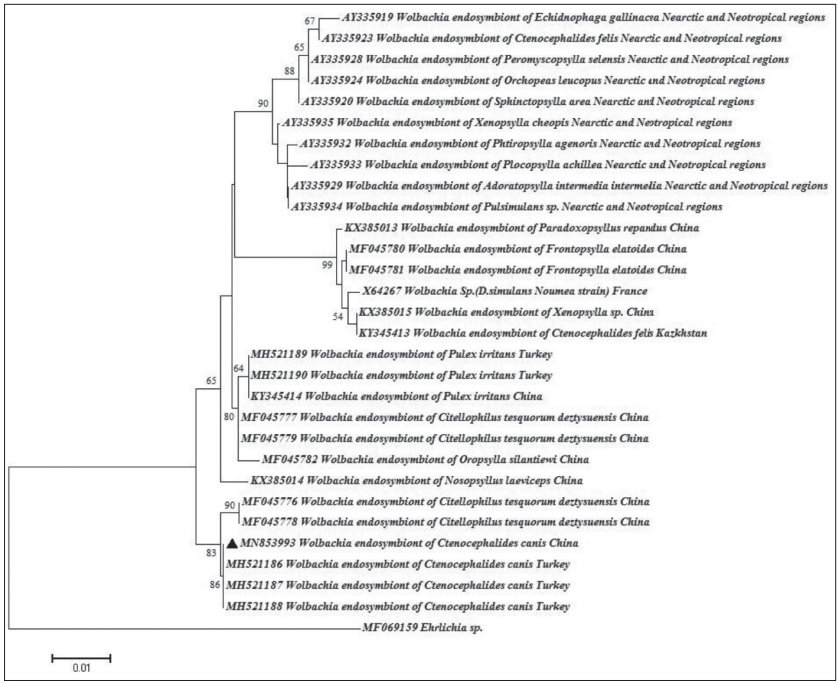

Fig 5. Phylogenetic tree of the *Wolbachia 16S rDNA* in *Ctenocephalides canis* from Eurasian lynx. The new sequences provided by the present study are indicated by a black triangle (containing the accession number). The phylogenic analyses were conducted using MEGA 6.0 software

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Letter to the Editor

Effect of Pimobendan in The Treatment of Pulmonary Hypertension in a Cat with Giant Right Atrium

(Dev Sağ Atriyumlu Bir Kedide Pimobendan'ın Pulmoner Hipertansiyon Tedavisindeki Etkinliği)

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Dear Editor,

Pulmonary hypertension (PH) is one of the most common health problem in human medicine, therefore many patients need anti-hypertensive medication to improve clinical signs and increase life quality and expectancy. In veterinary medicine, PH is associated with heartworm disease, pulmonary thromboembolism, chronic obstructive pulmonary disease, left-sided heart failure and large congenital cardiac shunts in dogs, but is rarely reported in cats [1-4]. Therefore, by this case presentation, we would like to share diagnostic algorithm and therapeutic approach of PH with veterinary academicians and practitioners to develop their skills on challenging disorders of feline cardiology.

The patient (Chinchilla, 3 years old, female, 4 kg) was referred from a private clinic to Veterinary Teaching Hospital with a history of lethargy, abdominal distention, exercise intolerance and abdominal breathing for a month. Patient with ascites and pleural effusion was already treated with furosemide and corticosteroid for 15 days. At admission, physical examination revealed 2/6-grade murmur from left and 4/6-grade at tricuspid valve puncta maxima. While heart (240 bpm) and respiratory rates (44 breath/min) increased, other clinical parameters were within normal range. Distended jugular vein was remarkable. Thoracic radiography revealed enlarged (right-sided) heart silhouette (VHS: 10.5), pulmonary artery (PA) bulging, dorsal deviation of trachea and caudal vena cava, decreased cardio-thoracic ratio in right side, and increased alveolar pattern. Pleural effusion and alveolar edema were minimal due to ongoing furosemide administration till admission. ECG examination showed sinus tachycardia. Complete blood cell count (Fuji VH5R) and serum biochemistry (Comprehensive Panel, Fuji Dri-Chem NX500I) were non-specific.

Transthoracic echocardiography was performed using standard imaging techniques with a 7.5-10 mHz phase array cardiac transducer (Caris-Plus Esoate) [1,3]. Two-dimensional right parasternal long and short-axis view revealed right atrial (RA) dilatation (2.8 cm) *(Fig.1-a)*, RA bulging into left atrium (LA), flattening of the interventricular septum (IVS) and right ventricular free wall (0.7 cm, ref.; 0.24±0.04 cm) and IVS thickness (0.94 cm, ref.; 0.5-0.9 cm) at diastole, suggestive for a giant RA and volume overload most probably due to PH and/pulmonary stenosis in this case. Since interatrial and IVS were intact and thicknesses and settlements of mitral and tricuspidal valves were normal, congenital defects that cause to RA dilation such as atrial and ventricular septal defects, Gerbode defect, endocardial cushion defect, and Ebstein's anomaly were excluded from the differential diagnosis list [1]. Geometric (LA:Ao ratio and left ventricle diameter and free wall thickness) and functional measurements such as fractional shortening were within reference limits, indicating that left-sided congestive heart failure was not a possible reason for increased volume overload in RA [2].

The main PA and its branches were dilated *(Fig.1-b)*. PA color flow Doppler showed the laminar flow as it should

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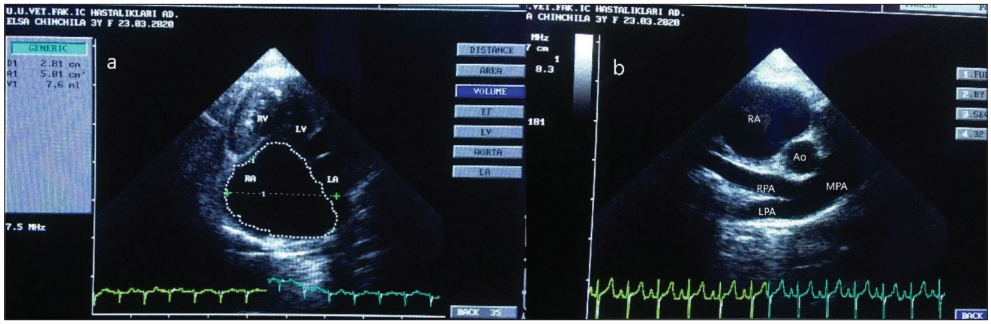

Fig 1. a) Enlarged right atrium (RA) in apical view (2.81 cm; 5.01 cm^2; 7.6 mL); **b)** Dilated main pulmonary artery (MPA; 2.0 cm) and right (RPA) and left branches (LPA) and dilated RA in right parasternal short axis-Aort (Ao; 1.0 cm) level

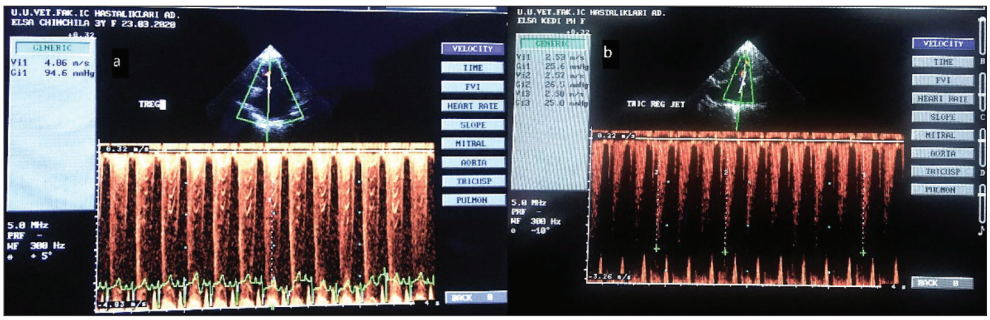

Fig 2. a) Pulse wave Doppler echocardiographic measurement of the tricuspidal regurgitant jet velocity and pressure gradient before (4.86 m/s and 94.6 mmHg) and **b)** after pimobendan treatment (2.53 m/s and 25.6 mmHg, respectively)

be, and its flow velocity and pressure gradient (PG) were within normal limits (0.9 m/s and 3.2 mmHg, respectively). Severe tricuspid regurgitation (TR) was observed without valvular pathology on apical 4-chamber view. Maximal TR jet velocity was 4.86 m/s and PG between right ventricle and RA was 94.6 mmHg *(Fig. 2-a)* according to Bernoulli equation [1,4]. That dogs with TR jet velocity >3.4 m/s have at least two echocardiographic signs of PH such as IVS flattening and RA and PA dilation (PA/Ao ratio: 2.0) increases the probability of PH [4]. PA systolic pressure (PASP) was 109.6 mmHg estimated by accepted formula (PASP= $4v^2$ + RA pressure) in compatible with severe idiopathic PH (type-I) in this case [2,3]. RA pressure was accepted as 15 mmHg for this case because RA dimeter (2.8 cm) was greater than LA (0.9 cm) [1]. Other possible reasons for PH which is left-sided heart failure (Type-II), respiratory diseases (Type-III), pulmonary embolism (Type-IV), parasitic diseases (Type-V), and multifactorial (Type-VI) were not possible for our case based on comprehensive diagnostic approaches [4].

The patient had supportive treatment for RA volume overload and PH with ramipril and hydrochlorothiazide combination (0.5 mg/kg, q24h, PO), furosemide (1 mg/kg, q24h, PO), Aspirin (10 mg/kg, q72h, PO) and dietary salt restriction [1,2]. Two weeks later, although clinical findings were improved slightly, the severity of TR jet velocity and PASP remained unchanged. Among treatment choices of PH, although sildenafil is the first option, there are some disadvantages due to its short half-life, difficulty of oral dosage in cats, and an expensive option for patient owners [4]. Instead, pimobendan, a particularly preferred drug in dogs, is a phosphodiesterase III inhibitor with calcium sensitizing effects thereby exerting positive inotropy and vasodilation. Pimobendan led to a short-term improvement in clinical signs and decreased TR in dogs [4], but there is not more information about this subject in feline practice. Considering the beneficial effects of pimobendan in dogs with PH [4], it was added to the standard treatment (0.25 mg/kg, q12h, PO). Three weeks after onset of pimobendan, TR jet was found to be lower (2.5 m/s) than previous one (4.8 m/s) *(Fig. 2-a,b)*, resulting in the full clinical improvement.

In conclusion, this presentation suggests that cases with isolated giant RA should be examined whether PH is present or not by transthoracic echocardiography. PASP could be estimated non-invasively by Doppler echocardiography in cats. Pimobendan therapy may have long-term clinical benefits which brings future possibilities of PH treatment which has not sufficient literature in feline medicine.

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Review

Neutrophils: A Critical Participator in Common Diseases of Ruminants

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Abstract

Neutrophils, pivotal effector cells involved in innate immunity, play a central role in various infectious and inflammatory diseases. Using a powerful phagocytic killing mechanism, these cells protect the host by destroying the invading pathogens. However, these cells can also cause varying degrees of tissue damage if their activation is not finely controlled. In recent years, the involvement of neutrophils in human diseases has been extensively studied, while their roles in ruminant diseases have rarely been investigated. In the present review, we mainly summarize current knowledge regarding the characteristics and functions of neutrophils in ruminants such as goats and cattle. We emphasize the involvement of these cells in several common diseases such as mastitis, Brucellosis, *Mycoplasma bovis* infection and parasitic infections, among others. We also focus on discussing the relevant mechanisms and signaling pathways underlying these observations. In addition, we compare the phenotypes and functions of neutrophils of different ruminant species. The studies about ruminant neutrophils should help elucidate the pathogenesis of many ruminant diseases and ultimately shed light on the development of novel therapeutics for these diseases.

Keywords: *Neutrophil, Protective immunity, Tissue damage, Ruminant disease, Immunotherapy*

Nötrofiller: Ruminantların Yaygın Hastalıklarında Kritik Katılımcı

Öz

Doğal bağışıklıkla ilişkili önemli efektör hücreler olan nötrofiller, çeşitli bulaşıcı ve inflamatuvar hastalıklarda temel rol oynarlar. Bu hücreler güçlü bir fagositik öldürme mekanizması kullanarak istilacı patojenleri yok eder ve konağı korur. Bununla birlikte, aktivasyonları iyi kontrol edilmezse değişen derecelerde doku hasarına neden olabilirler. Son yıllarda, nötrofillerin insan hastalıkları ile ilişkisi kapsamlı bir şekilde incelenirken, ruminant hastalıklarındaki rolleri nadiren araştırılmıştır. Bu derlemede esas olarak keçi ve sığır gibi ruminantlarda nötrofillerin özellikleri ve fonksiyonları ile ilgili güncel bilgileri özetlenmiştir. Bu hücrelerin mastitis, Bruselloz, *Mikoplazma bovis* enfeksiyonu ve paraziter enfeksiyonlar gibi yaygın görülen çeşitli hastalıklardaki önemi vurgulanmıştır. Ayrıca, ilgili mekanizmalar tartışılmış ve bu gözlemlerin altında yatan yollara işaret edilmiştir. Ek olarak, farklı ruminant türlerin nötrofillerinin fenotipleri ve işlevleri karşılaştırılmıştır. Ruminant nötrofilleri ile ilgili çalışmalar, birçok ruminant hastalığının patogenezinin aydınlatılmasına yardımcı olmalı ve sonuç olarak bu hastalıklar için yeni terapötiklerin geliştirilmesine ışık tutmalıdır.

Anahtar sözcükler: *Nötrofil, Koruyucu bağışıklık, Doku hasarı, Ruminant hastalığı, İmmünoterapi*

INTRODUCTION

Neutrophils are polymorphonuclear cells that act as the first line of defense against invading pathogens [1]. By rapidly combating intrusive microorganisms, they limit infections during the initiation stage of an immune response. Neutrophils are the most abundant leukocytes in the blood, where they complete their maturation after migration

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from the bone marrow to the vasculature [2]. Although the lifespan of most neutrophils is very short, with a circulating half-life of only 6-8 h, their overall number is stably maintained due to the dynamic balancing of production, retention, mobilization, margination and clearance [1]. Unlike most other immune cells, senescent neutrophils are an exceptionally long-lived population that may play an important role in maintaining neutrophil heterogeneity and homeostasis. They can be identified by their surface antigenic profile of $CXCR4^{high}$ $CD11b^{high}$ $CD62L^{low}$ and their uniquely small size and excessive nuclear lobulation [3].

Once neutrophils receive signals related to pathogen invasion and inflammation, they immediately migrate to sites where they are needed [1]. Neutrophils have long been considered the terminal effector cells of acute inflammatory response since they basically devour invading pathogens. In addition, numerous studies have shown that neutrophils also release effector molecules including various cytokines, extracellular traps and other effector mediators [2]. These effector molecules are closely tied to the activation, regulation and effector functions of both innate and adaptive immune cells [1]. Additionally, neutrophils can release granules containing highly toxic molecules, products of reactive oxygen species (ROS) and inflammatory cytokines, or may undergo NETosis to trap microbial invaders [4]. Consequently, if neutrophil-mediated immune responses are not strictly regulated, detrimental inflammations and host tissue damages can be caused as a result [5].

In recent years, ruminant farming has been threatened and impacted partly due to a lack of knowledge regarding invading pathogens and host responses. Because neutrophils are central to disease control and prevention, understanding their roles should be a priority. What has been known is that the morphology, function and quantity of neutrophils vary among different ruminant species [6]. Moreover, among healthy dairy goats or cattle, differences in the viability and morphology of blood and milk neutrophils have been reported [7].

In this review, we mainly describe the basic characteristics of neutrophils and review the specific roles of neutrophils in ruminants. We also highlight and summarize the roles of reciprocal communication pathways linking neutrophils with common ruminant diseases.

CHARACTERISTICS AND FUNCTIONS OF NEUTROPHILS

Neutrophil Chemotaxis

Neutrophils are the first immune cells to reach sites of inflammation and infection. They are recruited to such sites via chemotaxis, a cellular process depending on the extracellular chemoattractant gradient [8]. Neutrophils undergoing chemotaxis are polarized, whereby actin filament (F-actin)-based protuberances on their leading edges moving in a synchronized fashion with cytoplasmic contractions and the trailing edge myosin-based movements to move the entire cell forward. Neutrophil movement within a chemoattractant gradient relies on the action of G protein-coupled receptor (GPCR) signaling pathways employing formylated peptide receptors (FPR1/2/3), classical chemoattractant receptors (BLT1/2, PAFR and C5aR) and chemokine receptors (CXCR1/2 and CCR1/2) [1]. After a chemical attractant binds to a GPCR, a receptor conformational change occurs that results in the activation of downstream signaling pathways, including the phospholipase C (PLC) pathway. The PLC pathway activation triggers the production of diacylglycerol (DAG) and inositol triphosphate (IP3) that activate protein kinase C (PKC) and protein kinase D (PKD), which ultimately induce an increase in intracellular calcium level. PKC has multiple isoforms that interact with different participants to promote F-actin activity and regulate cofilin activity [9]. PKD, a direct effector of PLC/PKC axis proteins, can phosphorylate the cofilin phosphatase SSH2 to ultimately regulate downstream cofilin activity during GPCR-mediated neutrophil chemotaxis [10].

Cytotoxic Function

After neutrophils are recruited to sites of infection, they recognize and devour microbes. During this process, their cytotoxic function plays an important role in pathogen killing [8]. This function is made possible during neutrophil differentiation within the bone marrow whereby three types of granule proteins are formed in a stepwise fashion and assembled during maturation into a powerful pathogen-killing weapon. Meanwhile, within each neutrophil cytoplasm, numerous secretory vesicles are present that contain various types of plasma membrane receptors such as receptors for lipopolysaccharide (CD14), complement (CR1 and CR3/Mac-1), urokinase-type plasminogen activator, immune complex and chemoattractant (formyl peptide) [11]. At the infection site, neutrophil extracellular traps (NETs) also function as critical cytotoxins to promote "neutrophil apoptosis" through the process of "NETosis" to achieve extracellular entrapment of pathogens [4].

SPECIFIC FEATURES OF NEUTROPHILS IN RUMINANTS

Dairy Goat Neutrophils

During peak lactation, neutrophils in the blood and milk of dairy goats differ greatly in their morphological features and functions. Milk neutrophils are derived from migrating blood neutrophils that settle in the mammary gland, where they function to combat invading microbes that penetrate the physiological barriers of the papillary duct [12]. Occasionally, neutrophil band cells are found among blood neutrophils, but never among milk neutrophils. Notably,

milk neutrophils generally appear to be more mature than their blood counterparts. However, compared with blood neutrophils, milk neutrophils have impaired phagocytosis and oxidative burst functionality and lower viability, which may be due to spontaneous aging, interactions with milk components and/or diapedesis-based effects. Morphologically, milk neutrophils have a more ruffled appearance and possess a multi-lobed nucleus instead of the 2- to 3-lobed nucleus observed in the blood. In addition, milk neutrophils exhibit relatively lower ability to release gelatinase compared to blood neutrophils under both PMA stimulation or non-stimulation conditions [13].

In the 1960s, Paape introduced the term "somatic cells" (SCs) to refer to various types of cells found in mammalian milk [14]. As is well known, SCs are a handful of host cells found in animal milk that are predominantly leukocytes, including macrophages, neutrophils and lymphocytes, with a few epithelial cells. Thus, based on their origins, SCs can be categorized into two groups, blood-derived SCs and epithelial SCs [15]. The somatic cell count (SCC) in the milk of healthy dairy goats differs from that of cows, partially reflecting the differences in host processes that control normal mammary epithelial cell shedding and renewal [16]. Studies have shown that during mastitis, distinct neutrophil changes occur that differ between animal species, with neutrophils significantly outnumbering macrophages in the milk of mastitic sheep and goat, while macrophages predominate in healthy milk [17].

Bovine Neutrophils

Bovine neutrophils in the milk, similar to their goat counterparts, migrate from the blood to the mammary gland to provide the first line of defense against invading pathogens [7]. Although neutrophils newly migrating into the mammary gland are active phagocytic cells, they are continuously exposed to inhibitors in milk, such as fat globules and casein, resulting in decreased phagocytic capacity accompanied by morphological alteration [18]. Milk neutrophils possess large phagocytic vacuoles containing previously engulfed casein micelles with smooth surfaces and spherical shapes. These micelles are formed during loss of pseudopods by the internalization of pseudopod membrane material that results in milk fat globule formation.

Unlike other animal species in which neutrophils account for multitudinous blood leukocytes, bovine neutrophils make up only 25% of total blood leukocyte numbers. However, mature lactating Holstein cows have a potential pool of more than 100 billion circulating neutrophils that appear to be supplemented by a marginal pool of mature neutrophils adhering to vessel walls [19]. The makeup of neutrophil populations which respond to a particular mammary stimulus depends on the intensity of the stimulus and the strength of the chemotactic agent, with the number of neutrophils in bovine SCs determined by indirect immunofluorescence to be 3×10^4~3×10^6 cells/mL [20].

Akin to goat neutrophils, bovine neutrophils are also involved in the inflammatory process of mastitis. In fact, a wide variety of neutrophil β-defensins have been isolated that combat invasive pathogens [21]. However, these antimicrobial weapons also damage the fragile inner layer of the mammary gland and lead to permanent scar formation and decreased mammary epithelial cell participation in lactation. Interestingly, co-culture of neutrophils with cold-pressed terpeneless Valencia orange oil (TCO) has been shown to increase the chemotaxis of these cells *in vitro* without altering their phagocytic ability. Indeed, genes reflecting the pro-inflammatory immune response are generally down-regulated in the presence of TCO, resulting in the inhibition of bacterial growth without negatively altering neutrophil function [22], suggesting that TCO may serve as an effective therapy for mastitis.

Similarly, butyric acid, a short-chain fatty acid that can exert potent anti-inflammatory effects both *in vitro* and *in vivo*, could also serve as a mastitis treatment. Butyric acid acts via several mechanisms to regulate the innate immune response of ruminants: by activating neutrophils, inducing platelet activating factor (PAF), increasing CD63 expression, inducing the release of matrix metalloproteinase-9 (MMP-9) and lactoferrin, inducing NETs formation and through short-chain fatty acids (SCFA)-based pathways [23]. Previous studies have reported that increased production of SCFA is involved in subacute rumen acidosis and the activation of the inflammatory response [24]. In humans and rodents, SCFAs regulate the inflammatory response in the gut through free fatty acid receptor 2 (FFA2), which is activated by butyric acid in cattle. Researchers have found that butyrate activates bovine neutrophils to induce two second messenger events, Ca^{2+} influx and phosphorylation of mitogen-activated protein kinase (MAPK) that are involved in FFA2 activation. Butyric acid-induced Ca^{2+} influx is dependent on extracellular and intracellular Ca^{2+} sources and PLC activation. Therefore, butyric acid appears to be involved in SCFA regulation of inflammation through its effects on neutrophil activation [23].

RELATIONSHIP BETWEEN NEUTROPHILS AND COMMON DISEASES OF RUMINANTS

Role of Neutrophil Leucocyte in Cows and Goats During Mastitis

Mastitis is considered as one of the most complex diseases impacting dairy farming. The disease is caused by inflammation of the mammary gland initiated during several types of bacterial infections, among which *E. coli* infections, *M. agalactiae* infections and *S. aureus* infections are the most common ones [25]. Such infections cause damage or even necrosis of the mammary gland that eventually leads to low milk production and even the

removal of dairy production, seriously hindering the development of the livestock industry and imposing a great economic burden on farmers.

Studies about mastitis have shown that neutrophils can efficiently mount defenses against invading pathogens by migrating from the blood to the mammary gland, where they deploy a cascade of oxidative and non-oxidative response mechanisms to destroy pathogens [26]. In cows with mastitis, both SCC and milk neutrophil percentages are significantly increased while the viability of neutrophils is relatively low [27]. This phenomenon may result from pathogen-based signals that trigger both the release of neutrophils to blood from bone marrow and neutrophil migration from blood through the endothelial cell layer to the mammary gland, ultimately the increase of milk SCC. During this process, chemokine-mediated stimulation is a key determinant of SCC influx. Several potent chemo-attractants that recruit milk neutrophils include C5a as well as LPS, interleukin-6 (IL-6), IL-17 and IL-8 [26,28]. Although they are present in increased numbers, milk neutrophils possess decreased viability in mastitis. In a previous study, it was suggested that improving the viability of milk neutrophils might prevent or reduce the severity of *E. coli* mastitis in dairy cows [29]. In fact, the phagocytic activity of milk neutrophils is higher than blood neutrophils during subclinical and clinical mastitis, with the opposite observed in healthy cows [27]. However, when compared to healthy cows, blood and milk neutrophils from cows with clinical mastitis showed a significantly reduced phagocytic activity. These observations imply that the defense mechanism against invading pathogens of the mammary gland greatly depends on the rate at which neutrophils enter the infection site, their ability to produce reactive oxygen intermediates (ROI) and the number of circulating neutrophils at the infection site [30]. In healthy cows, milk neutrophils could be considered inactive cells when compared to circulating cells, since the phagocytic capacity of milk neutrophils is regulated by ROS production and milk neutrophils presumably undergo apoptosis to reduce ROI production after diapedesis. Nevertheless, the study found that immunosuppression always relied on cortisol, not on apoptosis, regardless of the physiological state of the cows [31] *(Fig. 1)*.

Blood neutrophils change their shape as they pass through the mammary epithelial barrier by becoming spherical. Upon entering the mammary gland, they become irregular milk neutrophils with wrinkled outer surfaces. The latter state not only helps to better internalize the cell membrane during phagosome formation, but also increases their surface areas for optimal phagocytosis [30] *(Fig. 1)*.

Bacteria have been shown to undermine neutrophil functions at each of these steps. In mastitis caused by *S. aureus*, IFN-γ serves as a neutrophil priming agent by acting as a primary agonist to influence the early effector arm of the neutrophil response and modulate post-response trafficking [32]. However, several researchers have found that *S. aureus* produces membrane-damaging peptides such as α, β, γ, δ-hemolysins, phenol soluble modulins (PSMs) and bi-component leukocidins that directly punch holes in immune cell membranes to lyse the cells [33]. Moreover, *S. aureus* employs numerous virulence factors to restrain neutrophil activation, chemotaxis and phagocytosis and target key host effector proteins. For example, extracellular fibrinogen-binding (Efb) protein and staphylococcal complement inhibitor (SCIN) proteins

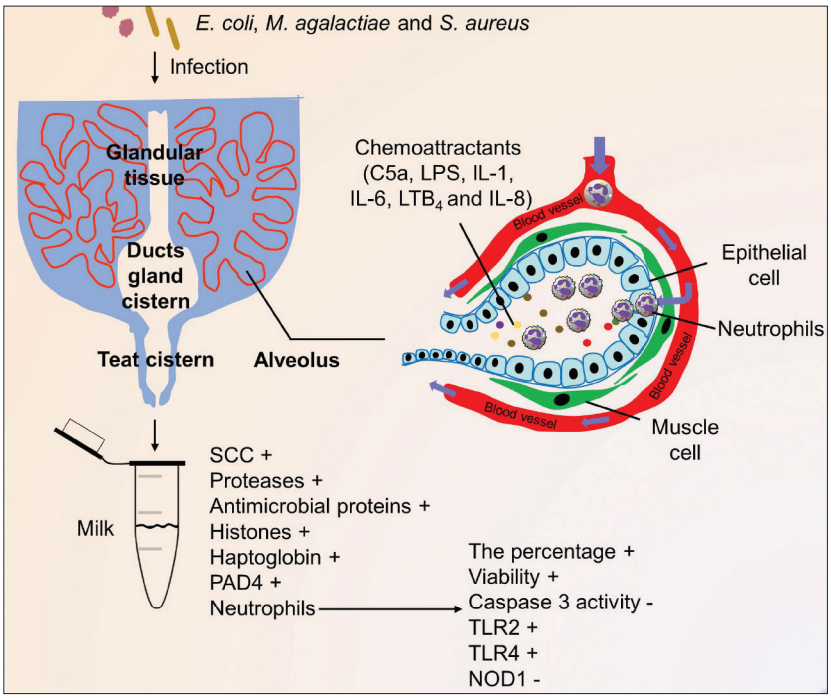

Fig 1. The role of neutrophils in mastitis. In glandular tissues affected by mastitis caused by *E. coli*, *M. agalactiae* and *S. aureus*, neutrophils patrolling in the bloodstream traverse the mammary endothelial cells and enter the mammary alveolus, where they destroy pathogens via oxidative and non-oxidative mechanisms. Neutrophils possess a wrinkled outer surface as they pass through endothelial cells. Within an alveolus, chemoattractants (C5a, LPS, IL-1, IL-6, LTB_4 and IL-8) tend to induce the participation of additional neutrophils and other immune cells during the inflammatory response. Changes in various milk proteins and cells are designated below the figure: "+" means increased or high expression; "-" means decreased or low expression

can target complement protein C3 convertase to prevent the formation of C3a and C3b, resulting in bacterial resistance to neutrophil phagocytosis [34]. Meanwhile, the *S. aureus* chemotaxis inhibitory protein (CHIPS) inhibits neutrophil migration and activation, preventing neutrophils from responding to both host- and bacterial-derived chemoattractants. Although toll-like receptor 2 (TLR2)-expressing cells can recognize staphylococcal glycan-related lipoproteins such as staphylococcal iron transporter C (SitC), *S. aureus* produces staphylococcus superantigen-like 3 (SSL3) protein that binds to TLR2 and inhibits the activation of neutrophils and other cells expressing TLR2. Crystal structure-based analysis demonstrates that the binding of SSL3 to TLR2 reduces the size of the available lipopeptide binding pocket by 50%, explaining the observed binding inhibition of TLR2 agonist Pam_2CSK_4. Moreover, *S. aureus* produces capsular polysaccharides and micro-capsules that may serve as yet another phagocytic escape strategy [35].

Abnormal apoptosis may also be a mechanism involved in neutrophil-based tissue damage in mastitis. Indeed, in the blood and milk of cows with mastitis, neutrophil caspase 3 activity is reduced, which may reflect reduced neutrophil apoptosis that promotes heightened neutrophil activity, ultimately causing tissue damage. Alternatively, the control of inflammation may involve extending neutrophil lifespan via the inhibition of NETosis [4]. In yet another possible scenario, higher neutrophil surface expression of TLR2 and TLR4, but not of TLR9, in cows with mastitis might enhance neutrophil pathogen recognition and immune responses, with crosstalk between C5a and neutrophil TLR4 signaling supporting a positive feedback loop that leads to severe mastitis responses *(Fig. 1)*. In this scenario, the onset of inflammatory reactions would somehow be linked to inefficient LPS detoxification that activates C5 cleavage to form C5a; if a large amount of C5a was produced, neutrophils would be activated via the C5a-C5aR pathway, a possible therapeutic target for mastitis treatment [36]. Another potential pathway in mastitis involves the interaction of neutrophils with the nucleotide-binding oligomerization domain-1 (NOD1), a key factor involved in the sensing of conserved bacterial peptidoglycan motifs that initiates pro-inflammatory and antimicrobial responses. In perinatal cows, neutrophil expression of NOD1 is decreased, which results in the inhibition of NOD1/NF-κB signaling, reduced neutrophil migration to *E. coli*-infected sites and impaired phagocytosis [37] *(Fig. 1)*. The NOD1/NF-κB pathway modulates neutrophil responses to reduce both neutrophil-mediated killing of bacteria and ROS production that in turn may control early inflammatory responses. Consequently, artificial restoration of neutrophil NOD1 function might be employed to either prevent or treat *E. coli*-induced mastitis.

Role of Neutrophil Leucocytes in Brucellosis

Brucellosis, caused by the genus *Brucella*, is a chronic global zoonosis [38]. Within infected macrophages and dendritic cells, pathogenic *Brucella* can replicate efficiently in the endoplasmic reticulum, a safe intracellular niche at the crossroads of many important host cell functions. There are many subspecies of *Brucella*, of which *B. melitensis*, *B. abortus* and *B. suis* are the three most common pathogens in humans and livestock [38]. Although *Brucella* spp. are closely related to each other genetically, they infect a broad range of livestock hosts including goats, cattle, camels, sheep, pigs and even wild animals such as bison, elk and feral swine [39]. Common symptoms of Brucellosis include high abortion rate, high mortality, infertility, low milk yield and a long interval between calving. The ability of *Brucella* spp. to evade the host immune system determines pathogen virulence, with one demonstrated mechanism involving escape from phagocytic killing. Thus, the mechanism by which neutrophils respond to pathogenic *Brucella* is a rather interesting and worthwhile research area.

Seminal studies have demonstrated that virulent smooth *B. abortus*, *B. melitensis*, *B. suis* and rough *B. canis* strains are resistant to neutrophil killing, regardless of whether they are resting or IFN-γ-activated. These results suggest that smooth strains may often be more virulent than rough strains. Bovine neutrophils counter infection by both smooth and rough *Brucella* spp. using oxidative burst-based killing [39]. This mechanism involves the inflammatory signaling regulator TAK1, a MAP3 kinase that is activated in response to cytokines, growth factors and TLR signals [39]. SYK kinase also plays a key role in the response of neutrophils to inflammations and is activated by Fc receptor binding. TAK1 is one of the major regulators of multiple kinase activation downstream of SYK in response to C-type lectin receptor stimulation. By inhibiting TAK1 or SYK, the degree of oxidative burst of neutrophils infected by *B. abortus* will be reduced, indicating a role of C-type lectin receptor in the response of bovine neutrophils to *B. abortus* infection [39] *(Fig. 2)*.

A *Brucella* virulence factor, β cyclic glucan (CβG), which has no toxicity for cells or animals, can induce dual pro-inflammatory and anti-inflammatory responses leading to transient neutrophil recruitment [40] *(Fig. 2)*. Notably, β-glucan is a polysaccharide of β-D-glucose extracted from cell walls of mushrooms, yeast, oats, barley, seaweed, algae and bacteria. Many researches and clinical studies have suggested that β-glucan acts as a biological response modifier that exhibits anti-tumor and anti-inflammatory properties. It is recognized by various pattern recognition receptors (PRR) expressed on dendritic cells, macrophages and neutrophils. In addition, complement receptor-3 (CR3), lactosylceramides, scavenger receptor and dectin-1 are also involved in β-glucan recognition, the outcome of which can trigger a series of signaling events that regulate the innate and the adaptive immune responses [41].

Effects of Mycoplasma bovis and Mycoplasma Lipoproteins on Neutrophils

Mycoplasma bovis (M. bovis) is the smallest bacterium

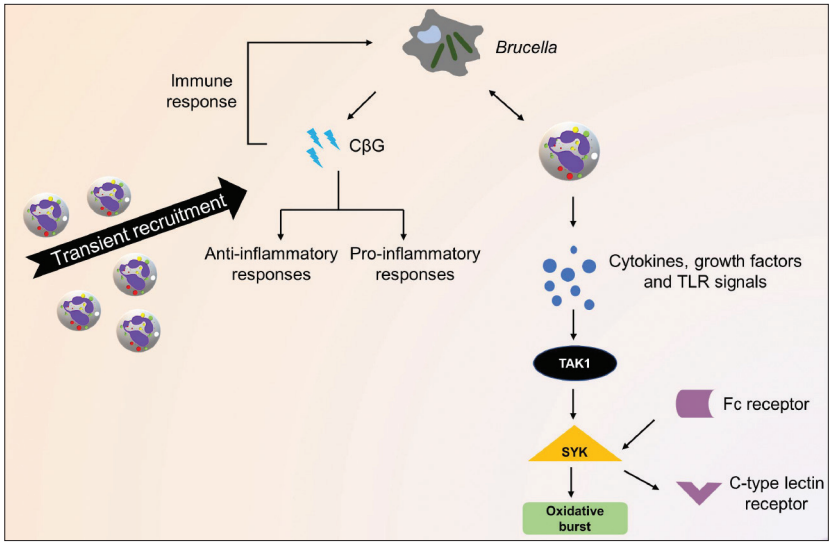

Fig 2. Neutrophils resist invasion by *Brucella*. *Brucella*-invading macrophages and other phagocytic cells interact with neutrophils and activate TAK1 in response to neutrophil TLR signals, as well as to multiple cytokines and growth factors. Downstream SYK is then activated by the action of the Fc receptor and further responds to signaling via the C-type lectin receptor. The neutrophil oxidative burst is eventually induced to resist *Brucella* infection. In addition, the virulence factor CβG produced by *Brucella* can induce transient neutrophil recruitment

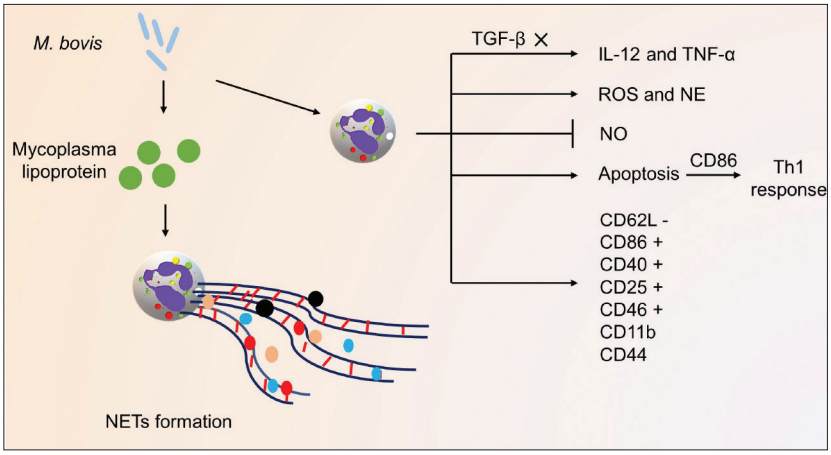

Fig 3. Effects of *M. bovis* and Mycoplasma lipoproteins on neutrophils. *M. bovis* invades the body and evades the immune response by accelerating neutrophil apoptosis and inducing ROS and NE production while inhibiting NO production. Subsequently, the Th1 response is induced by CD86-mediated signaling. During *M. bovis* infection, changes in molecules expressed on the neutrophil surface occur: "+" means increased or high expression; "-" means decreased or low expression; no mark means no change. Furthermore, neutrophils co-cultured with *M. bovis* could secrete IL-12 and TNF-α in the absence of TGF-β, while pathogen lipoproteins could induce the formation of neutrophil NETs

lacking a cell wall and often causes bovine chronic pneumonia and polyarthritis syndrome, conjunctivitis, otitis media, meningitis and mastitis [42]. These organisms require relatively rigorous cultural conditions for growth, posing a serious hindrance to vaccine preparation and disease prevention. Indeed, the lack of a vaccine has greatly influenced the health, welfare and productivity of dairy and beef cattle.

It has been shown that neutrophils exposed to *M. bovis* exhibit altered bactericidal function *in vitro* and that *M. bovis* inhibits the production of IFN-γ and TNF-α, but not IL-10 [43]. While little is known about how *M. bovis* evades host innate immunity, *M. bovis* has been shown to infect and persist within all PBMC subpopulations and erythrocytes [44]. Importantly, bacterial survival relies on the triggering of a series of pathogenic responses that delay the apoptosis of host cells. Such responses are carried out through the interaction of bacterial components with multiple proteases to activate cell survival pathways and prevent cytochrome C release. Conversely, *M. bovis* evades immune recognition by accelerating neutrophil apoptosis and inducing ROS production, while *in vitro* experiments have shown that the bacteria can also inhibit NO production, which has a dual biological role as a signaling molecule and cytotoxin. Meanwhile, IL-12 and TNF-α production in the absence of TGF-β has been observed when bovine neutrophils are infected with *M. bovis in vitro*. This observation suggests that an activation state is created based on inflammatory cytokines that may enhance the biological response of bovine neutrophils to *M. bovis* infection. In addition, host NE production is also important for the elimination of *M. bovis* *(Fig. 3)* [44].

As a unique survival mechanism during infection, *M. bovis* may drive neutrophils to a state of incompetence, as indicated by a decrease in CD62L expression with the up-regulation of CD86, CD40, CD25 and CD46 [44]. This mechanism appears to help *M. bovis* escape the host immune response and survive *in vitro* [44]. In this scenario, increased neutrophil apoptosis following *M. bovis* stimulation results in the activation of type 1 helper T cell responses, with increased expression of CD86 that is involved in antigen presentation to naive T cells [44] *(Fig. 3)*.

As another unique mechanism, mycoplasma lipoprotein, the most abundant component of the mycoplasma membrane, interacts with the host to promote cell

adhesion, determine strain virulence and induce NETosis. It is important that only fat-soluble mycoplasma proteins effectively induce NETs formation as an explanation for *M. agalactiae* evasion from NETs both in cultured sheep neutrophils and in mastitic mammary glands [45]. Using a different mechanism, *S. aureus* has been shown to induce macrophage apoptosis by disassembling NETs and converting them to deoxyadenosine, which induces caspase 3-mediated immune cell apoptosis [46]. In contrast, mycoplasma digests NETs DNA scaffolds, a mechanism requiring live bacteria that prevents the transmission of overlapping signals associated with neutrophil DNA and *M. agalactiae* within the mammary gland [45] *(Fig. 3)*.

Role of Neutrophil Leucocyte in Parasitic Infections

Parasitic infections are an enormous hazard to cattle and goat farming. Many studies have shown that NETs act as a novel effector mechanism in innate immunity against parasitic infections such as *Besnoitia besnoiti (B. besnoiti)*, *Eimeria arloingi (E. arloingi)* and *Cryptosporidium parvum (C. parvum)* infections in cattle and goats [47].

Cattle infected by *B. besnoiti* exhibit clinical symptoms such as systemic dermatitis, orchitis and vulvitis. *In vitro*, bovine neutrophils interact with *B. besnoiti* tachyzoites to induce rapid formation of NETs, which can be eliminated by DNase treatment or reduced by pre-incubation with NADPH oxidase inhibitors, neutrophil elastase (NE) and MPOs. It appears that neutrophils can immobilize parasites by forming an embedded structure so that other immune cells can be recruited quickly to synergistically kill the pathogen. *E. arloingi* coccidiosis in goats is mainly characterized by severe enteritis of 4- to 10-week-old goats, with infection rates as high as 100%. Once neutrophils contact *E. arloingi*, they form NETs during sporozoite or oocyst stages, whereby NETs effectively capture 72% of the sporozoites, greatly reducing the early infection rate [48]. *C. parvum* causes severe enteritis in neonatal livestock as well, triggering the formation of NETs in a time-dependent manner, whereby sporozoite-triggered NETs depend on intracellular Ca^{2+} concentration and ERK 1/2- and p38 MAPK-mediated signaling pathways. In fact, about 15% of parasites formed by *C. parvum* are immobilized in NETs [49].

Taken together, as the first line of immune defense, neutrophils play a unique role in parasitic infections. Neutrophils capture such pathogens mainly through the formation of NETs, delaying pathogen spread and promoting further elimination of invading parasites, although the detailed mechanisms are still unclear.

Other Modulators of Neutrophil Responses

In addition to diseases mentioned above, neutrophils also participate in other inflammatory ruminant diseases including bovine leukemia virus infection, *Pasteurella haemolytica* pneumonia, bovine respiratory disease, lung inflammation induced with *Mannheimia hemolytica*, pulmonary and systemic inflammation of fetal sheep and *Histophilus somni* infection, among others.

Host stress responses and viral invasion can adversely impact neutrophil responses and most typically affect neutrophil number without altering their recruitment, leukotoxin sensitivity or responses to bacterial infections. For example, non-cytogenetic bovine viral diarrhea virus infection leads to reduced production of neutrophils in the bone marrow and ultimately persistent neutropenia [50]. When occurring during abrupt weaning stress with increased neutrophil numbers, virus infections can recruits abundant neutrophils to sites of inflammation. However, the virus-triggered type I interferon response limits the production of CXC chemokines, leaving the host vulnerable to deadly secondary infections. Such infections include pulmonary *Streptococcus pneumoniae* or BRSV infections, the latter of which is characterized by neutrophil airway and alveolar infiltration with lower MPO levels and functionally immaturity [50].

Other factors that modulate neutrophil function include β-hydroxybutyrate,amediatorthatcanattenuateneutrophil phagocytosis and induce the formation of NETs [23]. Meanwhile, glucocorticoids can increase the survival and recruitment of neutrophils [50]. *H. somni* is able to inhibit an oxidative burst in both neutrophils and alveolar macrophages, while also inducing NETs production in a dose- and time-dependent manner that is not associated with lactate dehydrogenase release [50]. Taken together, both host and pathogen factors can significantly influence neutrophil function, with pathogens employing numerous mechanisms to escape killing by neutrophils and other cells of the immune system.

CONCLUSIONS AND PROSTECTS

We have discussed current knowledge regarding neutrophil chemotaxis and cytotoxic functions. We have also discussed specific features of ruminant neutrophils and the involvement of these cells in common diseases of ruminants. In recent years, studies revealing neutrophil origins, heterogeneity, circadian rhythms and epigenetic control of neutrophil activities have been widely discussed. However, most of these studies are based on humans or mice, researches on ruminant neutrophils are still lacking. Considering that neutrophils are actively involved in various ruminant diseases, further studies are required to explain and understand how neutrophils function during the progression of these diseases. In addition, how to better harness neutrophil activities during disease progression and how to optimally exploit these cells are also needed to explore in the future.

Conflict of Interest

The authors declare that no conflict of interest exists.

Author Contributions

W-T.M. and F.G. designed the structure of this article, S-L.N., F.G., C-X.Z., X-D.T. and M-J.L. drafted the first version of the manuscript, J-J.C, Y.W., D-K.C. and W-T.M. revised the manuscript.

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DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

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