ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Volume: 26 | Issue: 2 MARCH - APRIL | Year: 2020

ISSN: 1300-6045
e-ISSN: 1309-2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

JOURNAL OF THE FACULTY OF VETERINARY MEDICINE, KAFKAS UNIVERSITY

(MARCH - APRIL)

Volume: 26 Number: 2 Year: 2020

This journal is indexed and abstracted by Thomson Reuters Services beginning with Volume 13 (1) 2007 in the followings:

- **Science Citation Index Expanded (also known as SciSearch®)**
- **Journal Citation Reports/Science Edition**

This journal is also indexed and abstracted in:

- **Academic Search Premier**
- **CAB Abstracts**
- **DOAJ**
- **EBSCO**
- **Elsevier - Scopus**
- **EMBASE**
- **Essential Science Indicators - Zoological Record**
- **TÜBİTAK - ULAKBİM Yaşam Bilimleri Veri Tabanı**
- **Türkiye Atıf Dizini**
- **Veterinary Science Database**

YAZIŞMA ADRESİ (ADDRESS FOR CORRESPONDENCE)

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü
36040 - Kars / TÜRKİYE
Phone: +90 474 2426807-2426836/5228
Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

ELEKTRONİK BASKI (ELECTRONIC EDITION)
http://vetdergikafkas.org

ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)
http://submit.vetdergikafkas.org

Bu dergi Kafkas Üniversitesi Veteriner Fakültesi tarafından iki ayda bir yayımlanır
This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas

Kafkas Üniversitesi Veteriner Fakültesi Adına Sahibi (Owner)

Prof.Dr. Mete CİHAN
Dekan (Dean)

EDİTÖR (Editor-in-Chief)

Prof.Dr. İsa ÖZAYDIN

EDİTÖR YARDIMCILARI
(Associate Editors)

Prof.Dr. Özgür AKSOY
Prof.Dr. Duygu KAYA
Doç.Dr. Erol AYDIN
Doç.Dr. Ali YİĞİT
Dr.Öğr.Üyesi Ekin Emre ERKILIÇ

YABANCI DİL EDİTÖRÜ
(English Editor)

Prof.Dr. Hasan ÖZEN

İSTATİSTİK EDİTÖRÜ
(Statistics Editor)

Prof.Dr. İ. Safa GÜRCAN

BASKI (Print)
ESER OFSET MATBAACILIK
BOSNAHERSEK CAD. ALTUNALEM YAPI KOOP. ZEMİN KAT - ERZURUM
Tel: +90 442 2334667 E-mail: eserofset25@hotmail.com

EDİTÖRLER KURULU
(Editorial Board)

Prof. Dr. Harun AKSU, İstanbul University-Cerrahpaşa, TURKEY
Prof. Dr. Feray ALKAN, Ankara University, TURKEY
Prof. Dr. Kemal ALTUNATMAZ, İstanbul University-Cerrahpaşa, TURKEY
Prof. Dr. Divakar AMBROSE, University of Alberta, CANADA
Prof. Dr. Mustafa ARICAN, Selçuk University, TURKEY
Prof. Dr. Selim ASLAN, Near East University, NORTHERN CYPRUS
Prof. Dr. Sevil ATALAY VURAL, Ankara University, TURKEY
Prof. Dr. Tamer ATAOĞLU, İstanbul Medipol University, TURKEY
Prof. Dr. Levent AYDIN, Uludağ University, TURKEY
Prof. Dr. Les BAILLIE, Cardiff School of Pharmacy & Pharmaceutical Sciences, UK
Prof. Dr. K. Paige CARMICHAEL, The University of Georgia, USA
Prof. Dr. Ahmet ÇARHAN, Yıldırım Beyazıt Üniversitesi, TURKEY
Prof. Dr. Burhan ÇETİNKAYA, Fırat University, TURKEY
Prof. Dr. Recep ÇIBIK, Uludağ University, TURKEY
Prof. Dr. Ömer Orkun DEMİRAL, Erciyes University, TURKEY
Prof. Dr. İbrahim DEMİRKAN, Afyon Kocatepe University, TURKEY
Prof. Dr. Hasan Hüseyin DÖNMEZ, Selçuk University, TURKEY
Prof. Dr. Nazir DUMANLI, Fırat University, TURKEY
Prof. Dr. Emrullah EKEN, Selçuk University, TURKEY
Prof. Dr. Saeed EL-ASHRAM, Foshan University, CHINA
Prof. Dr. Marcia I. ENDRES, University of Minnesota, CFANS, USA
Prof. Dr. Ayhan FİLAZİ, Ankara University, TURKEY
Prof. Dr. Bahadır GÖNENÇ, Ankara University, TURKEY
Prof. Dr. Aytekin GÜNLÜ, Selçuk University, TURKEY
Prof. Dr. İ. Safa GÜRCAN, Ankara University, TURKEY
Prof. Dr. Ekrem GÜREL, Abant İzzet Baysal University, TURKEY
Prof. Dr. Johannes HANDLER, Freie Universität Berlin, GERMANY
Prof. Dr. Armağan HAYIRLI, Atatürk University, TURKEY
Prof. Dr. Ali İŞMEN, Çanakkale Onsekiz Mart University, TURKEY
Prof. Dr. M. Müfit KAHRAMAN Uludağ University, TURKEY
Prof. Dr. Mehmet Çağrı KARAKURUM, Mehmet Akif Ersoy University, TURKEY
Prof. Dr. Mehmet KAYA, Ondokuz Mayıs University, TURKEY
Prof. Dr. Ömür KOÇAK, İstanbul University-Cerrahpaşa, TURKEY
Prof. Dr. Marycz KRZYSZTOF, European Institute of Technology, POLAND
Prof. Dr. Ercan KURAR, Necmettin Erbakan University, TURKEY
Prof. Dr. Arif KURTDEDE, Ankara University, TURKEY
Prof. Dr. Hasan Rüştü KUTLU, Çukurova University, TURKEY
Prof. Dr. Erdoğan KÜÇÜKÖNER, Süleyman Demirel University, TURKEY
Prof. Dr. Levan MAKARADZE, Georgian State Agrarian University, GEORGIA
Prof. Dr. Erdal MATUR, İstanbul University-Cerrahpaşa, TURKEY
Prof. Dr. Mehmet NIZAMLIOĞLU, Selcuk University, TURKEY
Prof. Dr. Vedat ONAR, İstanbul University-Cerrahpaşa, TURKEY
Prof. Dr. Abdullah ÖZEN, Fırat University, TURKEY
Prof. Dr. Michael RÖCKEN, Justus-Liebeg University, GERMANY
Prof. Dr. Berrin SALMANOĞLU, Ankara University, TURKEY
Prof. Dr. Sabine SCHÄFER-SOMI, University of Veterinary Medicine Vienna, AUSTRIA
Prof. Dr. Çiğdem TAKMA, Ege University, TURKEY
Prof. Dr. Fotina TAYANA, Sumy National Agrarian University, UKRAINE
Prof. Dr. Zafer ULUTAŞ, Ömer Halisdemir University, TURKEY
Prof. Dr. Oya ÜSTÜNER AYDAL, İstanbul University-Cerrahpaşa, TURKEY
Prof. Dr. Thomas WITTEK, Vetmeduni Vienna, AUSTRIA
Prof. Dr. Rıfat VURAL, Ankara University, TURKEY
Prof. Dr. Hüseyin YILMAZ, İstanbul University-Cerrahpaşa, TURKEY

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

Abuzer TAŞ	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi
Ahmet ÇARHAN	Yıldırım Beyazıt Üniversitesi Tıp Fakültesi
Akiko UEMURA	Faculty of Veterinary Medicine, Tokyo University of Agriculture and Technology, Japan
Aksem AKSOY	Kafkas Üniversitesi Mühendislik Mimarlık Fakültesi
Alaeddin YÖRÜK	Osmaniye Korkutata Üniversitesi Ziraat Fakültesi
Ali YİĞİT	Kafkas Üniversitesi Veteriner Fakültesi
Aslı AKILLI	Ahi Evran Üniversitesi Ziraat Fakültesi
Asuman ÖZEN	Ankara Üniversitesi Veteriner Fakültesi
Aşkın YAŞAR	Selçuk Üniversitesi Veteriner Fakültesi
Atila Taner KALAYCIOĞLU	Karadeniz Teknik Üniversitesi Eczacılık Fakültesi
Ayla SOYER	Ankara Üniversitesi Mühendislik Fakültesi
Ayla ÜNVER ALÇAY	İstanbul Aydın Üniversitesi Anadolu BİL Meslek Yüksekokulu
Azize ŞENER	Marmara Üniversitesi Eczacılık Fakültesi
Bahri Devrim ÖZCAN	Çukurova Üniversitesi Ziraat Fakültesi
Banu YÜCEL	Ege Üniversitesi Ziraat Fakültesi
Behiç COŞKUN	Konya Gıda ve Tarım Üniversitesi Tarım ve Doğa Bilimleri Fakültesi
Burçak ASLAN ÇELİK	Siirt Üniversitesi Veteriner Fakültesi
Bünyamin TRAŞ	Selçuk Üniversitesi Veteriner Fakültesi
Cahit ÖZCAN	Siirt Üniversitesi Veteriner Fakültesi
Celal Şahin ERMUTLU	Kafkas Üniversitesi Veteriner Fakültesi
Cengiz ELMACI	Uludağ Üniversitesi Ziraat Fakültesi
Ceren ANLAŞ	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Çiğdem Eda BALKAN	Kafkas Üniversitesi Tıp Fakültesi
Dilek AKSU ELMALI	Mustafa Kemal Üniversitesi Veteriner Fakültesi
Dilek DÜLGER ALTINER	Kocaeli Üniversitesi, Turizm İşletmeciliği ve Otelcilik Yüksekokulu
Duygu DALĞIN	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Ebru YILDIRIM	Kırıkkale Üniversitesi Veteriner Fakültesi
Ender ERDOĞAN	Selçuk Üniversitesi Tıp Fakültesi
Evrim DERELİ FİDAN	Adnan Menderes Üniversitesi Veteriner Fakültesi
Fahrettin ALKAN	Selçuk Üniversitesi Veteriner Fakültesi
Faruk PEHLİVANOĞLU	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
Fatma Ceren ANLAŞ	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Ghassan ISSA	İstanbul Avrupa Meslek Yüksekokulu
Gökhan ERASLAN	Erciyes Üniversitesi Veteriner Fakültesi
Güzin KABAN	Atatürk Üniversitesi Ziraat Fakültesi
Handan ÇETİNKAYA	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Harun CERİT	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Hatice Özlem NİSBET	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Hilal BEDİR	Kafkas Üniversitesi Tıp Fakültesi
İbrahim DEMİRKAN	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
İlhami ÇELİK	Selçuk Üniversitesi Veteriner Fakültesi
İsmail Hakkı EKİN	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi
Jakub GRZESIAK	Wroclaw University of Environmental and Life Sciences, Poland
Kader YILDIZ	Kırıkkale Üniversitesi Veteriner Fakültesi
Kemal KARABAĞ	Akdeniz Üniversitesi Ziraat Fakültesi
Mehmet Cemal ADIGÜZEL	Atatürk Üniversitesi Veteriner Fakültesi
Mehmet GÜL	Atatürk Üniversitesi Veteriner Fakültesi
Mehmet İhsan SOYSAL	Namık Kemal Üniversitesi Ziraat Fakültesi
Meriç KOCATÜRK	Uludağ Üniversitesi Veteriner Fakültesi
Muhammed Enes ALTUĞ	Mustafa Kemal Üniversitesi Veteriner Fakülte

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

Murad GÜRSES	Balıkesir Üniversitesi Veteriner Fakültesi
Murat KARAHAN	Cumhuriyet Üniversitesi Veteriner Fakültesi
Musa KORKMAZ	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
Mustafa Selçuk ALATAŞ	Selçuk Üniversitesi Veteriner Fakültesi
Mükerrem KAYA	Atatürk Üniversitesi Ziraat Fakültesi
Nuriye Tuğba BİNGÖL	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi
Onur YILMAZ	Adnan Menderes Üniversitesi Ziraat Fakültesi
Özgür ÇADIRCI	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Özlem GÜZEL	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Pınar TATLI SEVEN	Fırat Üniversitesi Veteriner Fakültesi
Rabia Mehtap TUNCAY	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi
Raziye IŞIK	Namık Kemal Üniversitesi Ziraat Fakültesi
Sadık YAYLA	Dicle Üniversitesi Veteriner Fakültesi
Saira BASHIR	National Institute for Biotechnology and Genetic Engineering, Pakistan
Sefa ÇELİK	Afyonkarahisar Sağlık Bilimleri Üniversitesi Tıp Fakültesi
Sena ÇENESİZ	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Sevil ATALAY VURAL	Ankara Üniversitesi Veteriner Fakültesi
Sibel SİLİCİ	Erciyes Üniversitesi Seyrani Ziraat Fakültesi
Sinem ÜLGEN SAKA	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Şinasi UMUR	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Şule YURDAGÜL ÖZSOY	Adnan Menderes Üniversitesi Veteriner Fakültesi
Tuncay TUFAN	Siirt Üniversitesi Veteriner Fakültesi

İÇİNDEKİLER (Contents)

Kafkas Univ Vet Fak Derg
26 (2): 157-163, 2020
DOI: 10.9775/kvfd.2019.22582

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

The Effects of Physically Effective Neutral Detergent Fibre Content on Growth Performance and Digestibility in Beef Cattle Fed with Total Mixed Ration

Hıdır GÜMÜŞ [1,a] İsmail BAYRAM [2,b]

[1] Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, TR-15030 Burdur - TURKEY
[2] Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, TR-03200 Afyonkarahisar - TURKEY
[a] ORCID: 0000-0001-7077-1036; [b] ORCID: 0000-0002-9993-7092

Article ID: KVFD-2019-22582 Received: 02.05.2019 Accepted: 18.09.2019 Published Online: 24.09.2019

How to Cite This Article

Gümüş H, Bayram İ: The effects of physically effective neutral detergent fibre content on growth performance and digestibility in beef cattle fed with total mixed ration. *Kafkas Univ Vet Fak Derg,* 26 (2): 157-163, 2020. DOI: 10.9775/kvfd.2019.22582

Abstract

The objective of this study was to investigate the effects of physically effective neutral detergent fibre (peNDF) content on growth performance and digestibility in beef cattle fed with total mix ration (TMR). A total of 54 six-month-old male Holstein beef cattle (averaged weight of 280 kg) were divided into 3 groups each consisting of 18 cattle. Feed ingredients were added to the TMR wagon as follows; wheat straw, alfalfa hay, barley, corn, cotton seed meal, conventional beef feeding, corn silage, beet pulp, molasses and feed additives. TMR was offered daily to animals. The dietary treatments included; a) TMR diet mixed for 7 min (T1); b) TMR diet mixed for 14 min (T2), and c) free choice diet (FCD). The same ingredients feeds of TMR was given to the T1 and T2 groups but in different mixed times. Alfalfa hay and calf grower feed were separately offered animals in FCD. End of first month of trial, the daily feed intake (DFI) and dry matter intake (DMI) were significantly lower in the cattle that received FCD. At the end of the second month, the daily feed intake and dry matter intake were the highest in T1 diets among all the groups. There was no significant effect of different mixing times on n 48-h NDF digestibility (NDFD48) and ADF (ADFD48) digestibility of TMR. The lowest ration cost of 1 kg daily gain was observed for T2 and the daily feed intake cost was lower for FCD group than T1 and T2. It was concluded that mixing time had an effect on dry matter intake (DMI), average daily gain (ADG).

Keywords: *Feedlot cattle, In vitro digestibility, Particle size, peNDF, Total mix ration*

Toplam Karma Yemle Beslenen Besi Sığırlarında Fiziksel Etkin Nötral Deterjan Lifin Büyüme Performansına ve Sindirilebilirlik Üzerine Etkisi

Öz

Bu çalışmada toplam karma rasyon (TMR) ile beslenen besi sığırlarında fiziksel etkin nötral deterjan lifin (peNDF) büyüme performansına ve sindirilebilirlik üzerine etkisi incelenmiştir. Altı aylık yaştaki toplam 54 Holstein erkek besi sığırı (ortalama ağırlıkları 280 kg), her biri 18'er hayvandan oluşan 3 gruba ayrılmıştır. Yem bileşenleri TMR vagonuna; buğday samanı, yonca kuru otu, arpa, mısır, ayçiçeği küspesi, klasik besi yemi, mısır silajı, şeker pancarı posası ve katkı maddeleri sırası ile eklenmiştir. TMR hayvanlara günlük olarak sunulmuştur. Deneme grupları a) 7 dakika karışan TMR (T1); b) 14 dakika karışan TMR ve c) serbest seçenekli yemlemeden (SSY) oluşmuştur. T1 ve T2 grubuna verilen TMR'nin yem bileşenleri aynı iken karışma zamanları farklı olmuştur. Yonca kuru otu ve buzağı büyütme yemi SSY grubuna ayrı olarak sunulmuştur. Denemenin birinci ayı sonunda günlük yem tüketimi (GYT) ve günlük kuru madde tüketimi (GKMT) SSY grubunda önemli derecede düşük bulunmuştur. İkinci ayın sonunda günlük yem tüketimi ve kuru madde tüketimi T1 grubunda en yüksek bulunmuştur. TMR karıştırma süresinin 48 saat NDF (NDFD48) sindirimi ve 48 saat ADF (ADFD48) sindirimi üzerine etkisi olmamıştır. 1 kg canlı ağırlık artışı için rasyon maliyeti en az T2 grubunda saptanmış ve günlük yem tüketim maliyeti FCD grubunda T1 ve T2'ye göre düşük çıkmıştır. Bu çalışmada karıştırma süresinin kuru madde tüketimi, canlı ağırlık artışı üzerine etkisi olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Çiftlik hayvanı, In vitro sindirilebilirlik, Partikül boyutu, peNDF, Toplam karma rasyon*

INTRODUCTION

It is essential to improve management of agriculture and husbandry that will be very economical for the sustainability of the cattle industry. A large number of feeding systems has been used in feedlot management including total mix ration system, pasture system and conventional system [1]. Among these, TMR making is

İletişim (Correspondence)
+90 248 2132140 Fax: +90 248 2132001
hdrgumus@gmail.com; hgumus@mehmetakif.edu.tr

prepared to ensure balanced ration and a homogeneous ration for all the feed material [2]. The consistency of TMR can be dependent on many factors such as equipment condition, ingredient-mixing order, nutrient moisture and variability, which plays important roles in the production efficiency [3]. Total mix ration, or complete ration, is an important system for many feedlot performances e.g. daily gain (DG), feed intake and feed conversion ratio (FCR). TMR supplies the correct amount and a blend of balanced nutrients (energy/protein proportional) to cattle in a proper amount time. The advantages of TMR include that it allows cattle to consume the desired proportion of forages, increases feed efficiency, reduces risk of digestive upset and allows accuracy of diet formulation [4,5]. It is critical to point out that auditing of TMR must be controlled. The biggest problems are overfilling wagons, inadequate mixing time and improper loading of fluids while preparing TMR. Inadequate or extra-time mixing influences the feed particle size that stimulates rumination. The greater the amount of saliva they produce, the more their buffering capacity becomes [6]. It is possible to measure feed particle size that uses $peNDF_{\geq 4mm}$ and NDF content value of feeds [7]. $peNDF_{\geq 4mm}$ is the product of NDF concentration to the physical effectiveness factor (pef). Pef varies from 0 to 1. At 0 NDF, there is failure to stimulate chewing, and there is the maximum stimulation when Pef is 1 [8]. The Penn State particle Separator (PSPS) is being used at farms to determine the particle size and total mixed particles [7]. The model of PSPS consists of four screens with circular holes. When a TMR sample is analyzed with PSPS, four groups are formed; feed particle >19 mm (0.75 inch/upper sieve), feed particle >8 to 19 mm (0.31 inch/middle sieve), feed particles 4 mm to 8 mm (0.16 inch/lower sieve) and feed particles <4 mm (bottom pan). Poppi et al.[9] reported that feed particles retained on a 1.18-mm sieve had high resistance to passage from the rumen resulting in increasing chewing and rumination activity. Reduction of particle size increases the release rate from the rumen, and digestibility is reduced [7]. If the consistency of the ruminal mat is better, the passage of feed particles to the omasum is lower [8].

The objective of this study was to evaluate the effects of physically effective neutral detergent fibre content on live weight, dry feed intake, feed conversion ratio, daily gain, NDF and ADF digestibility in beef cattle.

MATERIAL and METHODS

This study was carried out from February to April in 2018 at a private feedlot farm in the province of Afyonkarahisar, located central Anatolia Turkey, 39° north latitude, 31° east longitude.

Experimental Unit

A total of fifty-four Holstein male beef cattle aged 6-7 months and weighed 280 kg were divided into 3 groups of 18 each in a generalized randomized block design based on their live weight. Before placing the male beef cattle to the stall, these animals were weighed on two consecutive days, and then, they were assigned to the groups. Accompanying the vaccination program, the study lasted 60 days, and among these days, the first 7 days constituted the adaptation period. The animals were kept in northside closed feeding pens kept in a shade area to protect them from north-east winds. The dimensions of the pens were 18 x 15 m, with 18 m^2 of concrete in front of the feed bunk. *Ad libitum* fresh water was provided during the experimental trial. The automatic float valve system was cleaned every week. A keystone was used as base that cleaned biweekly with a tractor. Light was provided from 18:00 h to 06:00 h in the pens throughout the study. The total mix ration was prepared as nutritional research council (NRC) requirements [10] by an expert in a horizontal De Laval wagon (12 m^3) with a digital weighing balance. The ration was formulated as monthly due to the variable nutrient requirement of beef cattle based on live body weight. The feed material was added to the TMR wagon as follows; wheat straw, alfalfa hay, barley, corn, cotton seed meal, conventional beef feeding, corn silage, beet pulp, molasses and feed additives. After adding all the ingredients to the TMR wagon, it was mixed for 7 min to prepare the T1 ration and 14 min to prepare the T2 ration. The TMR was offered daily to the animals for feeding. The dietary treatments included; a) TMR diet mixed for 7 min (T1); b) TMR diet mixed for 14 min (T2), and c) free choice diet (FCD). The same TMR ration was given to the T1 and T2 groups but in different times. The FCD group ration consists of alfalfa hay and calf grower feed that were separately offered to the animal. The FCD ration did not mixed in TMR wagon. The animals were fed twice a day in the morning (08:00) and evening (18:00). The feeds were delivered by more than 5-10% to the bunk needed for dry matter intake to ensure an *ad libitum* system. The residual of feeds given the other cows in farm, which was not in the experiment. times. The animals had free access to mineral blocks at all. DFI was measured by weighing feed offered and residue left over with 24 h during the study. FCR was calculated individually as LWG:DMI (kg of live weight gain divided by kg of DMI). The beef cattle were weighed by using a digital weighing machine 2 h before feed delivery at the beginning and every 4^{th} week during the entire experimental period. The average daily gain (ADG) of each cattle was determined by dividing live weight gain by the number of days on feed.

Chemical Analyses and Digestibility

The feed samples were analyzed based on the methodology of the Association of Official Analytical Chemists (AOAC) [11] for DM (method 934.01), ash (method 942.05), ether extract (EE) (method 920.39) and N (method 954.01) contents. NDF and ADF were determined according to the method described by Goering and van Soest [12]. Crude fibre

content was determined by the methods of Crampton and Maynard [13]. Non-fibrous carbohydrates (NFC) were calculated by difference NFC= 100 – (%NDF + %CP + %Fat + %Ash) according to the standards of the National Research Council [10]. Forty-eight-h *in vitro* true NDF and ADF digestibility ($NDFD_{48}$ and $ADFD_{48}$) values were determined using a Daisy II Incubator (Ankom Technology, NY, USA) described by Vogel et al.[14]. Approximately 0.5 g of each sample was put into F57 fibre bags (ANKOM Technology, NY, USA) and heat-sealed. The samples were placed into a digestion jar with two buffers and rumen fluid (Buffer A: KH_2PO_4, $MgSO_4$-$7H_2O$, NaCl, $CaCl_2$-$2H_2O$, and Urea; Buffer B: Na_2CO_3 and Na_2S-9H20). Rumen fluid was collected and mixed at Afyon Kocatepe University Animal Research Center from two cannulated nonlactating Brown Swiss that were fed a forage-based diet (60:40 forage:concentrate). After the inclusion of the rumen fluid, all jars were flushed with CO_2 and placed into a preheated incubator (39°C). The incubation process was continued for 48 h with agitation. After the incubation process, the samples were rinsed with cold tap water for about 10 min. Then, the $aNDF_{om}$ and ADF_{om} procedures were performed in a way previously described for Fibretherm FT12 (Gerhardt GmbH&Co. KG, Königswinter, Germany). The digestibility of each sample was then determined via weight differences before and after digestion.

Particle Size Analysis

The particle sizes of TMR were determined by using PSPS. The model of PSPS consisted of four screens with circular holes sized 19 mm (0.75 inch/Upper), 8 mm (0.31 inch/ Middle sieve), 4 mm (0.16 inch/lower sieve) and a bottom pan. Each TMR sample of about 1000 g was placed on the top of the PSPS box. On a flat surface, we shook the PSPS in the north-south direction 5 times, then rotated the box by a one-fourth turn. This series was repeated 8 times, for a total of 40 shakes so that the box was shaken 5 times for each set. The residual of particles in each sieve were weighted on digital scales. The values obtained in each sieve were recorded to calculate the physical effectiveness factor (pef) which was determined by adding particle size retained on the three boxes (19-8-4 mm). The $peNDF_{\geq 4mm}$ content of TMR was calculated by multiplying the neutral detergent fibre (NDF) content of TMR by pef [15]. The proportion of sample DM collected in the ≥4 mm sieve was commonly used as the physical effectiveness factor in the equation [16]. The particle sizes of TMR were determined to repeat 4 replicates per sample and average the results have a representative sample of TMR.

Statistical Analysis

The statistical analyses were carried out with SPSS (Statistical Package for the Social Sciences; Inc., Chicago, IL, USA). All data were subjected to statistical analyses using one-way ANOVA except for digestibility data. The differences among the groups were calculated using Duncan's test [17]. The *in vitro* NDFD and ADFD values of each TMR were evaluated using PROC T-TEST of SAS version 8.3 (SAS Institute Inc., Cary, NC, USA) after log-transforming the digestibility levels. The level of significance was taken as $P<0.05$ for all data.

RESULTS

The Ingredients and chemical composition of the total mix rations (T1 and T2) are presented in *Table 1*. The effects of $peNDF_{\geq 4mm}$ content on growth performance in male Holstein beef cattle are presented in *Table 2*. At the beginning of the study, the live weights were 218.52, 210.11 and 199.44 kg in T1, T2 and FCD, respectively. The live weights were 260.88, 264.72 and 243 kg in T1, T2 and FCD, respectively at the end of 30th days. At the end of the first month, the daily feed intake and dry matter intake were significantly lower ($P<0.05$) in the cattle that received FCD (7.57, 6.69 kg/day) than in those that received T1 (10.46, 8.12 kg/day) and T2 (11.03, 8.57 kg/day). Daily weight gain value was found as 1.41, 1.81 and 1.45 kg for the T1, T2 and FCD diets, respectively. The FCR value was 8.12, 8.57 and 6.69 for T1, T2 and FCD, respectively. There were significant differences among the treatments in terms of daily feed intake, dry matter intake and feed conversion ratio ($P<0.05$). The final live weights were 298.82, 308.61 and 286.11 kg in T1, T2 and FCD, respectively, at the end of the study. At the end of the second month, the daily feed intake and dry matter intake values were the highest in the T1 diets among all the groups *(Fig. 1)*. The mean daily feed intake and dry matter intake were very similar in T1 and T2 (12.78, 9.59; 12.77, 9.58), and these values were lower in the FCD (8.80, 7.77) group. The daily weight gain was the highest in T2 (1.46) followed by FCD (1.44) and T1 (1.26). FCR was lower in the FCD diets in comparison to the other diets.

In the first month *(Table 3)*, the proportion remaining on the upper part (19 mm of sieve size) in T1 was higher than that in T2. The percentage of particles retained on the 19-mm sieve decreased by increasing the mixing time of TMR. Forage particle size reduction resulted in increased DMI

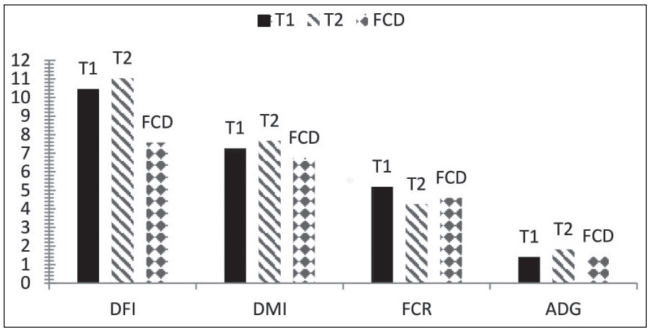


Fig 1. Growth performance in Holstein beef cattle. **DFI:** Daily feed intake; **DMI:** Dry matter intake; **FCR:** Feed conversion ration; **ADG:** Daily gain; **T1:** Total mix ration was mixed 7 min; **T2:** Total mix ration mixed 14 min; **FCD:** The animals was offered feed separetely

Table 1. *The ingredients and chemical composition of total mix ration*

Diet Formulation/Months	Amount of Supplementation, kg as a Feed Basis	
	First Month	Second Month
Corn silage	0.50	1.00
Wheat straw	0.80	0.84
Alfalfa hay*	1.00	1.25
Barley	0.80	1.20
Corn	0.70	1,00
Sunflower oil	1.50	1.70
Molasses	0.40	0.40
Sugar beet pulp	1.00	1.35
Calf grower feed**	2.50	2.54
Limestone	0.05	0.05
Salt	0.02	0.02
Vitamin- mineral premix[1]	0.01	0.01
Concentrate: forage ratio	64.43;35.56	60.91;39.08
Chemical composition as DM	**First month**	**Second month**
Dry matter	77.70	76.38
Crude protein	18.37	17.17
Ether extract	4.61	4.30
Crude fibre	17.61	17.14
Ash	7.32	6.93
Nötral detergent fibre	32.49	32.04
Acid detergent fibre	20.53	15.26
Hemicellulose	11.96	16.78
[2]Non-fibre carbohydrate	37.21	39.56
[3]Nitrogen free extract (NFE)	52.09	54.56

*[1] Each kilogram of vitamin-mineral mix contains 12.000.000 IU A vit, 20.000 mg E vit, 50.000 mg Mn, 50.000 mg Fe, 50.000 mg Zn, 10.000 mg Cu, 800 mg I, 150 mg Co, 150 mg Se; [2] NFC= 100 – (%NDF + %CP + %EE + %Ash); [3] NFE= 100- (CP+CF+EE+Ash); * Alfalfa hay: DM: 89.92; CP 16.65; EE: 2.35; CF: 21.18; Ash: 9.52; NDF: 41.15; ADF: 29.95; ** Calf grower feed: DM: 90.51; CP 19.21; EE: 3.76; Ash: 5.02; NDF: 18.35; ADF: 9.38*

(T1: 8.12; T2: 8.57 kg/d). The percentage of the particles retained on the middle part (8 mm of sieve size) was 33.59 in T1, which was higher in comparison to T2 (23.91). The fraction of particles retained in the lower part (4 mm of sieve size) was 47.11and 48.12 in T1 and T2, respectively. The percentage of particles obtained in the bottom sieve decreased in parallel by increasing the mixing time of TMR (T1: 14.66; T2: 14.18). In the second month *(Table 3)*, the proportion remaining on the upper part (19 mm of sieve size) in T1 was higher than that in T2. The percentage of particles retained on the 19-mm sieve decreased by increasing the mixing time of TMR (T1: 13.51; T2: 8.28). Similar DMI values were observed in T1 (9.58 kg/d) and T2 (9.57 kg/d). The percentage of particles retained on the middle part (8 mm of sieve size) was 25.78 in T1, which was quite similar in T1 in comparison to T2 (26.61). The fraction of particles retained in the lower part (4 mm of sieve size) was 41.94 and 34.70 in T1 and T2, respectively, and it was

Table 2. *Effects of peNDF$_{\geq 4mm}$ content on growth performance in Holstein beef cattle ration*

Groups*	First Month		
	T1[1]	T2[2]	FCD[3]
Initial live weight (kg)	218.52±4.99	210.11±6.30	199.44±3.87
Live weight (kg, 30th day)	260.88±4.46	264.72±4.97	243±4.31
Daily feed intake (kg/d)	10.46±0.64^{a}	11.03±0.16^{a}	7.57±0.20^{b}
Dry matter intake (kg/d)	8.12±0.50^{a}	8.57±0.12^{a}	6.69±0.18^{b}
Feed conversion ratio (DMI/DG) kg/kg	5.80±0.35^{a}	4.76±0.06^{b}	4.56±0.12^{b}
Daily gain (kg)	1.41	1.81	1.45
Groups*	**Second Month**		
	T1	**T2**	**FCD**
Final live weight (kg, 60th day)	298.82±5.10	308.61±4.46	286.11±4.40
Daily feed intake (kg/d)	12.78±0.22^{a}	12.77±0.64^{a}	8.80±0.38^{b}
Dry matter intake (kg/d[4])	9.59±0.17^{a}	9.58±0.48^{a}	7.77±0.34^{b}
Feed conversion ratio (DMI/DG) kg/kg	7.57±0.13^{a}	6.53±0.33^{b}	5.55±0.24^{c}
Daily gain (kg)	1.26	1.46	1.44

[1] T1 = Total mix ration was mixed 7 min; [2] T2 = Total mix ration was mixed 14 min; [3] FCD = The animals was offered feed separately; [4]Total mix ration dry matter is of 77.70 in 1th month; Total mix ration dry matter is of 75.06 in 2th month; Concentrate dry matter is of 87.75; Alfalfa dry matter is 89.95
^{a,b,c} Means with different superscript in the same row are different (P<0.05)

higher than the recommended values [16]. The percentage of particles obtained in the bottom sieve increased in parallel by increasing the mixing time of TMR (T1: 26.93; T2: 27.47).

For all months, there was no significant effect of different mixing times on either *in vitro* 48-h NDF digestibility ($NDFD_{48}$) or ADF ($ADFD_{48}$) digestibility of TMR. However, $NDFD_{48}$ values of the rations mixed for 14 min were numerically higher than those mixed for 7 min for first and second months. $ADFD_{48}$ values of the ration mixed for 7 min were numerically lower than those mixed for 14 min for both months *(Table 4)*.

In this study, daily feed intake cost was found as $ 1.857, 2.120 and 1.847, whereas the ration cost of daily gain was $ 1.528, 1.290 and 1.397 for T1, T2 and FCD, respectively *(Table 5)*.

DISCUSSION

Growth performance and digestibility are essential factors that are related to the physical effectiveness of a

Table 3. *Particle size distribution of the total mixed (%)*

Size of Sieve	Groups			
	T1		T2	
	Proportion Remaining On Each Sieve %	Compute Cumulative Percentage Undersized[1]	Proportion Remaining On Each Sieve %	Compute Cumulative Percentage Undersized
First Month				
19 mm	11.45±0.58	100	7.86±0.51	100.00
8 mm	33.59±1.12	88.55	23.91±2.12	92.14
4 mm	47.11±1.46	54.96	48.42±3.05	68.23
Bottom Pan	14.66±1.00	7.85	14.18±1.79	19.81
[1]$pef_{\geq 4mm}$	0.92		0.80	
NDF (DM %)	32.04		32.04	
[2]$peNDF_{\geq 4mm}$	29.47		25.63	
Second Month				
19 mm	13.51±2.49	100.00	8.28±0.58	100.00
8 mm	25.78±0.82	86.49	26.61±2.02	91.72
4 mm	41.94±2.23	60.71	34.70±1.97	65.11
Bottom Pan	26.93±1.23	18.77	27.47±1.54	30.41
[1]$pef_{\geq 4mm}$	0.82		0.7	
NDF (DM %)	32.49		32.49	
[2]$peNDF_{\geq 4mm}$	26.65		22.74	

[1] Cumulative percentage undersized refers to the proportion of particles smaller than a given size. For example, on average, 95% of feed is smaller than 0.75 inches, 55% of feed is smaller than 0.31 inches and 35% of feed is smaller than 0.16 inches; [1] The pef is calculated as sum of the proportion of particles retained on both 19.0; 8.0-and 4 mm sieves; [2] The $peNDF_{\geq 4mm}$ was calculated multiplying the pef by the NDF content of the TMR

Table 4. *The NDF[1] and ADF[2] digestibility of different total mixed rations (%)*

Item		T1	T2	SEM	P-value
$NDFD_{48}$ [3]	1st month	42.0240	43.1287	3.0389	0.7500
	2nd month	44.1678	46.7243	3.7482	0.5326
$ADFD_{48}$ [4]	1st month	84.7536	86.3563	2.1267	0.4930
	2nd month	85.0954	86.7157	2.2642	0.5138

[1] Amylase-treated, ash-free aNDFom; [2] Ash-free ADFom; [3] NDF Digestibility (48-h in vitro incubation), % of NDF; [4] ADF Digestibility (48-h in vitro incubation), % of ADF

Table 5. *Economic analyses of ration*

Groups	Daily Feed Intake Cost	Ration Cost of 1 kg Daily Gain
T1	10.92±0.36 TRY/$1.857	8.27±0.42 TRY/$1.528
T2	11.19±0.22 TRY/$2.120	6.98± 0.41 TRY/$1.290
FCD	10.79±0.32 /$1.847	7.56±0.28 TRY/$1.397

Current prices were used in economic analyses. 1 $ is 5.41 TRY (13.11.2018). Price of TMR is first month: 0.961 TRY/kg; Price of TMR is second month: 0.923 TRY/kg; Price of calf rower feed is 1.4 TRY/kg; Price of alfalfa hay is 0.95 TRY/kg

ration or feeding ingredients. Excessive amount of long fibres could limit dry matter intake and digestibility, as a short particle size decreases chewing activity and results in a decline of saliva production and rumen pH [18]. Especially regarding this concern, several studies have been conducted on dairy cows [8,19,20], beef cattle [7,21] and goats [22].

In this study, in the first month, the dry matter intake (DMI) in the FCD group was significantly lower than those in the other groups ($P<0.05$). The PeNDF value did not affect dry matter intake, but the feed conversion ratio was lower ($P<0.05$) in T2 in comparison to T1. DMI increased numerically (T1: 8.12; T2: 8.57) with decreasing dietary $peNDF_{\geq 4mm}$ (T1: 29.47; T2: 25.63). The results were consistent

with those found by Park et al.[23], who reported that dry matter intake was increased significantly with respect to reduced peNDF$_{\geq 4mm}$. There seems to be a relationship between particle size and DMI. A study by Allen [24] reported that decreased particle size decreases the filling effects of forage and increases the ruminal passage rate. Feed intake may be reduced due to a long particle size that occupies larger volumes per unit of DM weight in the rumen content. In contrast to the other studies [7], DMI was increased linearly by increasing mixing time. According to the results of this study, the daily weight gain of the T2 diets was significantly ($P<0.05$) lower than those in the other groups, which was in accordance with another study by Jang et al.[22] Feed conversion ratio (T1: 5.80; T2: 4.76 kg/kg) was decreased linearly by decreased peNDF$_{\geq 4mm}$ (T1: 29.47; T2: 25.63). The results obtained from this study were in compliance with those found by Oh et al.[7], who reported that feed conversion ratio was decreased by increasing the peNDF$_{\geq 4mm}$ content.

In this study, increased final live weight gain (T1: 298.82; T2: 308.61; FCD: 286.11) resulted in cattle fed T2 having higher daily weight gain than the other groups (T1: 1.26; T2: 1.46; FCD: 1.44).

In the first month, the percentage of particles retained on the 19-mm sieve, 8-mm sieve, 4 mm sieve and bottom pan of the T1 and T2 groups were 11.45, 7.86; 33.59, 23.91; 47.11, 48.42 and 14.66, 14.18; respectively. Kononoff and Heinrichs [15] recommended for high production dairy cows for the particles in the upper sieve to be 2-8%, 30 to 50% in the middle sieve, 10 to 20% on the 4-mm sieve and no more than 30 to 40% in the bottom pan. In this study, the peNDF$_{\geq 4mm}$ values (T1: 29.47; T2: 25.63) and feed conversion ratios (T1: 5.80; T2: 4.76) were decreased linearly by increasing mixing time.

The reduction in the feed conversion ratio might be related to an increase in forage surface area for the microbial attack of the rumen [25], and it causes increased fermentation [26]. Based on the results from a previous study [7], increasing revolution per min (T1: 12.000 rpm; T2: 15.000 rpm) was attributed to reduction in peNDF$_{\geq 4mm}$ value (T1: 21.71; T2: 16.22).

In the second month, the percentages of particles retained on the 19-mm sieve, 8-mm sieve, 4 mm sieve and bottom pan of the T1 and T2 groups were 13.51, 8.28; 25.78, 26.61; 41.94, 34.70 and 26.93, 27.47 respectively. The mixing time (T1: 7 min; T2 14 min) affected the peNDF$_{\geq 4mm}$ value (T1: 26.65; T2: 22.74) and feed conversion ratio (7.57; 6.53). This result was consistent with those found by Oh et al.[7], who reported that the proportion of particles retained on the 19-mm sieve (T1: 14.15; T2: 5.81; T3 1.81) decreased by increasing the mixing time (T1: 3 min; T2: 10 min; T3: 25 min) of TMR. Likewise, feed conversion ratio and feed intake were influenced by peNDF$_{\geq 4mm}$ of TMR along with the NDF contents of forages [8]. Additionally, feeding high NDF in TMR resulted in gut filling effect (bulkiness) in relation to the voluntary intake of the reticulorumen [9,27] with decreasing digestibility [28,29], thereby decreasing feed intake [30,31]. Wang et al.[32], reported that roughage particle size in the diet did not significantly affect the DMI; this could be attributed to a result of the lower roughage percentage (50% DM for forage and silage) in the diet. Possibly, the cattle may prefer to consume longer forage to ensure the sufficient rumen fill or to increase their foraging needs [33].

Although NDF and ADF digestibility values of the diets mixed for 14 min were numerically higher than those mixed for 7 min for both months, the duration of mixing had no significant effect on NDFD$_{48}$ or ADFD$_{48}$. In earlier studies, researchers observed higher rumen passage rates with smaller particle sizes, and they predicted a possible decrease on fibre digestibility in this manner [17,33,34]. However, more recently, Yansari et al.[35] showed that reducing forage particle size had no effect on the digestibility of ADF in mid-lactation dairy cows. This was in agreement with our results. Furthermore, the researchers observed no effect of particle size on the digestibility values of most nutrients such as dry matter, organic matter, non-fibre carbohydrates or crude protein in the same study. On the other hand, Yansari et al.[34] interestingly observed a lower NDF digestibility value for smaller forage particle sizes, contrary to our findings. As it is well-known, increasing DMI is encouraged for the passage rate of digesta in the gastrointestinal tract [25]. In our study, the observed effects of particle sizes on DMI were expected to decrease fibre digestibility. However, contrary to our expectations, no significant effect was observed on fibre digestibility with different particle sizes. Although the 48-h *in vitro* ADF and NDF digestibility model had no kinetic passage rate effect unlike the other kinetic *in sacco* and *in situ* methods, the effects of forage particle size on digestibility might be more relevant for the rate of passage rather than the direct rate of digestibility. This hypothesis might explain the significant effects on DMI and lack of effects by particle size on NDF and ADF digestibility without an outflow rate.

The current prices for diets were used to calculate daily feed intake cost and ration cost. In this study, daily feed intake cost was found as $1.857, 2.120 and 1.847, whereas the ration cost of daily gain was $1.528, 1.290 and 1.397 for T1, T2 and FCD, respectively. The estimated daily feed intake cost was quite similar for the T1 ($1.857) and T2 ($2.120) groups, but the ration cost of 1 kg daily gain in T2 ($1.290) was lower than that in T1 ($1.528) due to high average daily gain (T1: 1.33; T2: 1.63). Small particle sizes had a direct effect on feed intake and daily gain, thereby decreasing ration costs for 1 kg of daily weight gain.

In conclusion, the optimal (standard) value ranges were determined (%) for dairy cattle but not for beef cattle. Statistically significant or insignificant differences were mostly due to individual differences in animals such as age or sex of the animal, physically effective fibre content

of the forage and ration ingredient. It is concluded that mixing time is important for dry matter intake, daily gain.

REFERENCES

1. Oelberg TJ, Stone W: Monitoring total mixed rations and feed delivery system. *Vet Clin North Am Food Anim Pract,* 30, 721-744, 2014. DOI: 10.1016/j.cvfa.2014.08.003

2. Costa A, Agazzi A, Perricone V, Savoini G, Lazzari M, Nava S, Tangorra FM: Influence of different loading levels, cutting and mixing times on total mixed ration (TMR) homogeneity in a vertical mixing wagon during distribution: A case study. *Italian J Anim Sci,* 18, 1093-1098, 2019. DOI: 10.1080/1828051X.2019.1618742

3. Sova AD, LeBlanc SJ, McBride BW, DeVries TJ: Accuracy and precision of total mixed rations fed on commercial dairy farms. *J Dairy Sci*, 97, 562-571, 2014. DOI: 10.3168/jds.2013-6951

4. Linn JG: Management of TMR feeding Programs. Dairy Uptade Issue, 121, 7-15, 1995.

5. Lammers BP, Heinrichs AJ, Ishler VA: Use of total mixed rations for dairy cows. PennState Extension Issue, DAS 94-25; 2013.

6. Moya D, Mazzenga A, Holtshausen L, Cozzi G, Gonzalez LA, Calsamiglia S, Gibb DG, McAllister TA, Beauchemin KA, Schwartzkopf-Genswein K: Feeding behavior and ruminal acidosis in beef cattle offered a total mixed ration or dietary components separately. *J Anim Sci*, 89, 520-530, 2011. DOI: 10.2527/jas.2010-3045

7. Oh MR, Hong H, Li HL, Jeon BT, Choi CH, Ding YL, Tang YJ, Kim EK, Jang SY, Seong HJ, Moon H: Effects of physically effective neutral detergent fibre content on intake, digestibility, and chewing activity in fattening heifer fed total mixed ration. *Asian Australas J Anim Sci*, 29, 1719-1724, 2016. DOI: 10.5713/ajas.16.0344

8. Zebeli Q, Aschenbach JR, Tafaj M, Boguhn J, Ametaj BN, Drochner W: Role of physically effective fibre and estimation of dietary fibre adequacy in high-producing dairy cattle. *J Dairy Sci*, 95, 1041-1056, 2011. DOI: 10.3168/jds.2011-4421

9. Poppi DP, Minson DJ, Ternouth JH: Studies of cattle and sheep eating leaf and stem fractions of grasses. I. The voluntary intake, digestibility and retention time in the reticulorumen. *Crop Pasture Sci*, 32, 99-108, 1981. DOI: 10.1071/AR9810099

10. NRC: Nutrient Requirements of Beef Cattle. 7th Rev. ed., National Academy Press, Washington, DC, USA; 2000.

11. AOAC: Official Methods of Analysis. Association of Official Analytical Chemists. AOAC International. 15th ed., Arlington, VA, USA; 1990.

12. Goering HK, Van Soest PJ: Forage Fibre Analysis. Agric Handbook No, 379. Washington, DC (Agricultural Research Service) US, Department Agriculture, 1970.

13. Crampton EW, Maynard LA: The relation of cellulose and lignin content to nutritive value of animal feeds. *J Nutr*, 15, 383-395, 1938. DOI: 10.1093/jn/15.4.383

14. Vogel KP, Pedersen JF, Masterson SD, Toy JJ: Evaluation of a filter bag system for NDF, ADF, and IVDMD forage analysis. *Crop Sci*, 39, 276-279, 1999. DOI: 10.2135/cropsci1999.0011183X003900010042X

15. Kononoff PJ, Heinrichs AJ: The effect of reducing alfalfa haylage particle size on cows in early lactation. *J Dairy Sci,* 86, 1445-1457, 2003. DOI: 10.3168/jds.S0022-0302(03)73728-X

16. Allen MS, Mertens DR: Evaluation constraints on fibre digestion by rumen microbes. *J Nutr*, 118, 261-270, 1988. DOI: 10.1093/jn/118.2.261

17. Dawson B, Trapp RG: Basic and Clinical Biostatistics. 3rd ed., Lange Medical Books/McGraw-Hill Medical Publishing Division, New York, USA, 89 (2): 131-153, 2001.

18. Gonzales FHD, Olmo DMD, Muino R, Benedito JL, Hernandez J, Castillo C, Pereira V: Feed sorting and intake affected by the physical form and composition of the total mixed ration in dairy cows. *Rev Bras Saude Prod Anim*, 16 (3), 736-745, 2015. DOI: 10.1590/S1519-99402015000300023

19. Tafaj M, Maulbetsch A, Zebeli Q, Steingass H, Drochner W: Effects of physically effective fibre concentration of diets consisting of hay and slowly degradable concentrate on chewing activity in mid lactation dairy cows. *Arch Anim Nutr*, 59, 313-324, 2005. DOI: 10.1080/17450390500247840

20. Yang WZ, Beauchemin KA: Effects of physically effective fibre on chewing activity and ruminal pH of dairy cows fed diets based on barley silage. *J Dairy Sci*, 89, 217-228, 2006. DOI: 10.3168/jds.S0022-0302(06)72086-0

21. Liu YF, Sun FF, Wan FC, Zhao HB, Liu XM, You W, Cheng HJ, Liu GF, Tan XW, Song EL: Effects of three feeding systems on production performance, rumen fermentation and rumen digesta particle structure of beef cattle. *Asian Australas J Anim Sci*, 29, 659-665, 2016. DOI: 10.5713/ajas.15.0445

22. Jang SY, Kim EK, Park JH, Oh MR, Tang YJ, Ding YL, Seong HJ, Kim WH, Yun YS, Moon SH: Effects of physically effective neutral detergent fibre content on dry matter intake, digestibility, and chewing activity in Korean native goats *(Capra hircus coreanae)* fed with total mixed ration. *Asian Australas J Anim Sci*, 30, 1405-1409, 2017. DOI: 10.5713/ajas.16.0868

23. Park JH, Kim KH, Park PY, Jeon BT, Oh MR, Jang SY, Sung SH, Moon SH: Effects of physically effective neutral detergent fibre content on dry matter intake, digestibility and chewing activity in beef cattle fed total mixed ration. *Anim Prod Sci*, 55, 166-169, 2014. DOI: 10.1071/AN14241

24. Allen MS: Effects of diet on short-term regulation of feed intake by lactating dairy cattle. *J Dairy Sci,* 83, 1598-1624, 2000. DOI: 10.3168/jds.S0022-0302(00)75030-2

25. Kim DH, Choi SH, Park SK, Lee SS, Choi CW: Effect of corn grain particle size on ruminal fermentation and blood metabolites of Holstein steers fed total mixed ration. *Asian Australas J Anim Sci*, 31 (1): 80-85. 2018. DOI: 10.5713/ajas.17.0069

26. Zadeh AB: Investigation of ruminants digestibility based on the particle size of nutritional materials. *Bull Environ Pharmacol Life Sci,* 3, 59-62. 2014.

27. Mertens DR: Predicting intake and digestibility using mathematical-models of ruminal function. *J Anim Sci,* 64, 1548-1558, 1987. DOI: 10.2527/jas1987.6451548x

28. Boudon A, Peyraud JL, Faverdin P, Delagarde R, Delaby L, Chaves AV: Effect of rumen fill on intake of fresh perennial ryegrass in young and mature dairy cows grazing or zero-grazing fresh perennial ryegrass. *Animal*, 3, 1706-1720, 2009. DOI: 10.1017/S1751731109990486

29. Ahmad S, Jabbar MA, Khalique A, Saima F, Shahzad N, Ahmad M, Fiaz M, Younas U: Effect of different levels of NDF on voluntary feed intake, dry matter digestibility and nutrients utilization ın dry nili ravi buffaloes. *J Anim Plant Sci*, 24, 1602-1605, 2014.

30. Esmaeili M, Khorvash M, Ghorbani, GR, Nasrollahi SM, Saebi M: Variation of TMR particle size and physical characteristics in commercial Iranian Holstein dairies and effects on eating behaviour, chewing activity, and milk production. *Livest Sci,* 191, 22-28, 2016. DOI: 10.1016/j.livsci.2016.07.003

31. Shaver RD, Nytes AJ, Satter LD, Jorgenson NA: Influence of feed intake, forage physical form, and forage fibre content on particle size of masticated forage, ruminal digesta, and feces of dairy cows. *J Dairy Sci,* 71, 1566-1572 1988. DOI: 10.3168/jds.S0022-0302(88)79720-9

32. Wang HR, Chen Q, Chen LM, Ge, RF, Wang MZ, Yu LH, Zhang J: Effects of dietary physically effective neutral detergent fiber content on the feeding behavior, digestibility, and growth of 8- to 10-month-old Holstein replacement heifers. *J Dairy Sci,* 100, 1161-1169, 2017. DOI: 10.3168/jds.2016-10924

33. Greter AM, Prinsen M, Duffield TF, McBride BW, Widowski TM, DeVries TJ: Growing dairy heifers prefer supplementary long straw when fed a nutrient-dense ration in a limited amount. *J Dairy Sci,* 96, 3950-3958, 2013. DOI: 10.3168/jds.2013-6625

34. Mertens DR: Creating a system for meeting the fibre requirements of dairy cows. *J Dairy Sci,* 80, 1463-1481, 1997. DOI: 10.3168/jds.S0022-0302(97)76075-2

35. Yansari AT, Valizadeh R, Naserian A, Christensen DA, Yu P, Shahroodi FE: Effects of alfalfa particle size and specific gravity on chewing activity, digestibility, and performance of Holstein dairy cows. *J Dairy Sci,* 87, 3912-3924, 2004. DOI: 10.3168/jds.S0022-0302(04)73530-4

Kafkas Univ Vet Fak Derg
26 (2): 165-169, 2020
DOI: 10.9775/kvfd.2019.22636

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Colonoscopic Diagnosis of Atresia Coli in Calves: 19 Cases (2016-2018) [1]

Taylan ÖNYAY [1,a] Kamil Serdar İNAL [1,b] Birsen Deniz ÖZBAKIR [1,c]
Hatice Özlem NİSBET [1,d] Cenk YARDIMCI [1,e] Kamil SAĞLAM [1,f] Ahmet ÖZAK [1,g]

[1] *The study was presented as an oral presentation in the II[nd] International and XVI[th] National Veterinary Surgery Congress of Turkey, 20-23 September 2018, Bafra, North Cyprus*

[1] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Surgery, TR-55200 Atakum/Samsun - TURKEY
[a] ORCID: 0000-0001-5638-7257; [b] ORCID:0000-0003-0690-5743; [c] ORCID: 0000-0001-9832-1256; [d] ORCID: 0000-0001-9981-1193
[e] ORCID: 0000-0001-6126-3603; [f] ORCID: 0000-0003-4467-187X; [g] ORCID:0000-0002-4359-0691

Article ID: KVFD-2019-22636 Received: 14.05.2019 Accepted: 27.09.2019 Published Online: 27.09.2019

How to Cite This Article

Önyay T, İnal KS, Özbakir BD, Nisbet HÖ, 1,D Yardimci C, Sağlam K, Özak A: Colonoscopic diagnosis of atresia coli in calves: 19 cases (2016-2018). *Kafkas Univ Vet Fak Derg,* 26 (2): 165-169, 2020. DOI: 10.9775/kvfd.2019.22636

Abstract

Atresia coli is sporadically seen in calves and lethal unless intestinal patency is restored through surgery. While there are some reports of surgically corrected animals with atresia coli giving birth to healthy offspring, there is still controversy about treating such cases, since survival is not guaranteed and there is a chance of tainting the herd's gene pool. This study aims to investigate the efficacy of colonoscopy in cases of atresia coli in determining the atresia type and evaluating the lumen. Nineteen calves of different breeds and sex between the ages of 1 to 15 days were included in the study. Colonoscopy duration, maximum distance of the endoscope from the anus and any discernible changes inside the lumen were recorded for each case. The technique was easy to use, required no anaesthesia or sedation and very informative about the condition of the mucosa. Definitive diagnosis of the atresia type using colonoscopy is not possible unless a complete blind end is seen and iatrogenic damage may be caused during the procedure. We believe its diagnostic use can provide the surgeon with data to select which procedure to use and help with prognosis.

Keywords: *Atresia coli, Calf, Colonoscopy*

Buzağılarda Atresia Coli'de Kolonoskopik Tanı: 19 Olgu (2016-2018)

Öz

Atresia coli, buzağılarda sporadik olarak gözlenir ve cerrahi olarak bağırsaktaki içeriğin geçişi sağlanmadığı sürece ölümcüldür. Atresia coli olan ve tedavi edilen hayvanlardan sağlıklı yavru elde edildiğine dair yayınlar olsa da, tedavi sonrası sağ kalım garantisi olmadığından ve sürünün gen havuzunu kirletme riski bulunduğundan böyle hastaların tedavisi halen tartışmalıdır. Bu çalışmada, atresia coli olan buzağılarda kolonoskopi kullanımıyla atresia coli tipinin belirlenmesi ve lümenin değerlendirilmesini amaçlanmıştır. Çalışmada 1 ila 15 günlük yaş arasında, farklı ırk ve cinsiyette 19 buzağı değerlendirilmiştir. Her olgu için kolonoskopi süresi, endoskop ile anüs arasındaki uzaklık ve lümen içerisindeki gözlenebilir değişiklikler kaydedilmiştir. Tekniğin kolay olduğu, anestezi veya sedasyonsuz uygulanabildiği ve mukozanın durumunu değerlendirmede yararlı olduğu belirlenmiştir. Bunun yanında, doğrudan kör uç görülmediği sürece atresia tipinin kesin olarak belirlenmesi mümkün değildir ve uygulama sırasında iatrojenik hasar oluşabilmektedir. Tanıda kolonoskopi kullanmanın, uygulanacak cerrahi girişim şekline karar verilmesi ve prognozun belirlenmesinde katkı sağlayacağına inanıyoruz.

Anahtar sözcükler: *Atresia coli, Buzağı, Kolonoskopi*

INTRODUCTION

Atresia coli is one of the most commonly seen malformations in calves. The disorder is traditionally classified in 4 types [1] but only the first three types of atresia are commonly seen [2]. First type is stenosis, with or without an intact or perforated diaphragm, second is cord atresia and the third is blind end atresia [2]. Etiology of this condition has been theorized to involve disruption of the blood supply to the bowels through local or genetic factors, thus causing stenosis or atresia [3]. Early rectal palpation have been said to cause the malformation [4]

İletişim (Correspondence)
+90 362 3121919/1195
taylan.onyay@omu.edu.tr

although there is also evidence to the contrary, suggesting that early palpation is safe [5,6]. Genetic inheritance of this disease is proposed, and there is proof of this for some breeds, like Holstein-Friesian [7], but it remains suspect in many others [2,3]. The clinical and epidemiological prevalence suggests that this condition may not be inherited in non-predisposed breeds [4].

The prognosis of this condition is poor and worsens with the passage of time [8], as the calves born with this malformation are incompatible with life unless intestinal patency is restored with surgery [1,4]. Affected calves have been known to produce healthy offspring after being surgically corrected [9].

The diagnosis of atresia coli was traditionally based on clinical examination and findings alone. Radiography have been used for diagnosis [10] and there is a report of using contrast radiography and ultrasonography [11] to diagnose the condition. Definitive diagnosis is made by exploratory laparotomy [12].

Colonoscopy is a minimally invasive method that could potentially help to the diagnosis of the condition and provides data in preoperative planning. This study aims to evaluate the viability of this technique in calves with atresia coli.

MATERIAL and METHODS

The study material consisted of calves that were brought to the Teaching Hospital of Animal Hospital of Ondokuz Mayıs University, Faculty of Veterinary Medicine, and Department of Surgery between the years of 2016 and 2018. Animals that were unable to defecate since birth were included in the study regardless of breed and gender.

After learning patient history from the owners, physical examination of each patient was made in accordance to our routine patient exam protocol (which included heart rate, respiratory rate, capillary refill time, dehydration level, body temperature). The hemogram of each patient was evaluated from a blood sample taken from the jugular vein. The patient was then placed on lateral recumbence, rectum was lubricated with polyacrylamide gel and colonoscopy was performed without sedation or anaesthesia. A 1.4 m long flexible endoscope with a diameter of 8.5 mm (Aohua, LG-200) was used for colonoscopy. The endoscopies were recorded using an external TV card's (AverTV) own media capture software.

The atresia types were only estimations based on endoscopic findings since diagnosis requires visualizing the intestines from the outside. If there was no mucosal wall or blind end to be seen and the endoscope could not be moved further due to narrowing of the lumen, the condition was classified as "suspected type I atresia". Although the lumen was stenotic, the condition was presumed to be atresia since no feces were present. If the canal tapered to a very narrow point where the endoscope could not fit through it was recorded as "suspected type 3" atresia, and if a blind end could be clearly visualized it was recorded as a "type 3" atresia.

Breed, gender, atresia type, colonoscopy duration, reach of the endoscope (length from the anus to the maximum possible colonoscopic exam site) and mucosal damage due to previous procedures (done by the owner or private veterinarians) or during the colonoscopy performed in our hospital were recorded for each patient. The presence of iatrogenic damage during colonoscopy was determined when the endoscope was being retracted, if the mucosa was intact upon entry but damaged and/or bleeding while retracting, it was considered to be iatrogenic damage.

RESULTS

The most common breed was Simmental (n=12) followed by Jersey (n=1), Holstein mix (n=4), Limousin (n=1) and Holstein (n=1). Ten calves were male and 9 were female. Ages of the calves at the time of presentation were between 2 and 15 (mean 5.31) days. The colonoscopy is considered to be completed when the endoscope could not be moved any further due to narrowing or upon encountering a blind end. The duration varied between 1'49" and 7'57" (4'53" on average).

There was some amount of mucus content in each colon that was evaluated; in the early cases, that were brought between 1-3 days had clear, bright mucus over their mucosa, the mucus was thicker and yellow in older (4-6 days old) cases and the last three calves (7, 13 and 15 days old) had very thick, almost solid chords or lumps of dried mucus in their lumen. Types of colon content are shown in *Fig. 1*.

Twelve calves were recorded as having "suspected type 1 atresia", 3 with type 3 and 4 with suspected type 3 atresia *(Fig. 2)*. Mucosal damage was seen in 16 calves during colonoscopy; 12 cases had varying degrees of damage due to previous catheterization attempts with various materials. Six of these were delayed cases and had complications (Cases 12, 13, 14, 16, 18, 19), 5 were acute (Cases 2, 6, 7, 11, 17) being brought to the hospital right after the attempts with only superficial injury to the mucosal lining. In one case, the catheterization attempt of the owner resulted in perforation of the blind end (Case 9). The iatrogenic damage with colonoscopy was caused in 4 cases (Cases 3, 8, 10, 15) by either pushing the endoscope without sufficient lubrication of the lumen or inserting it too forcefully. Iatrogenic damage caused either erythematous changes or superficial abrasions and bleeding on the colon mucosa. Examples of mucosal damage were shown in *Fig. 3*.

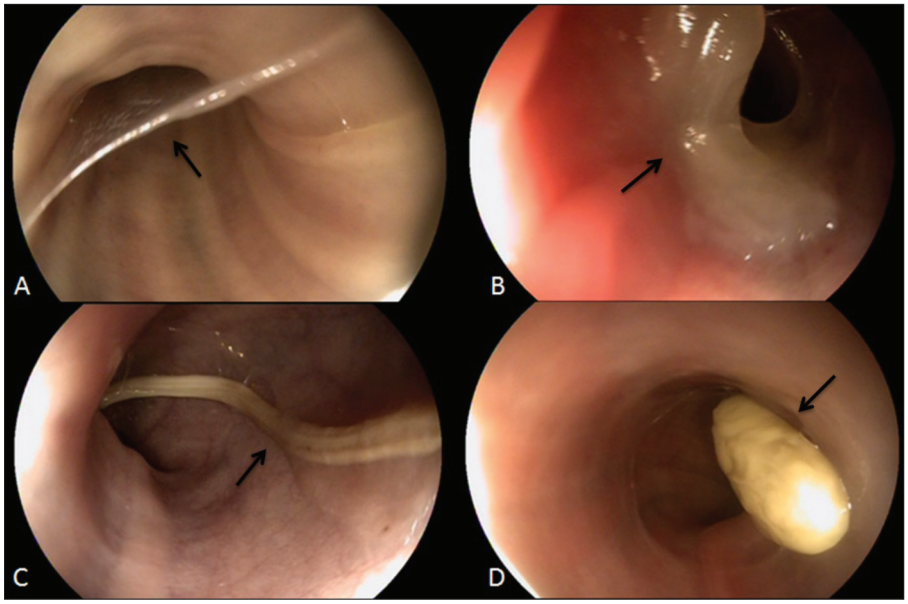


Fig 1. Types of mucus seen in the colon lumen *(black arrows):* (A) One day old clear and bright mucus (B) More opaque, gelatinous mucus (C) Thick, hardened chord of dried mucus (D) and the "lump" like appearance of the colon content

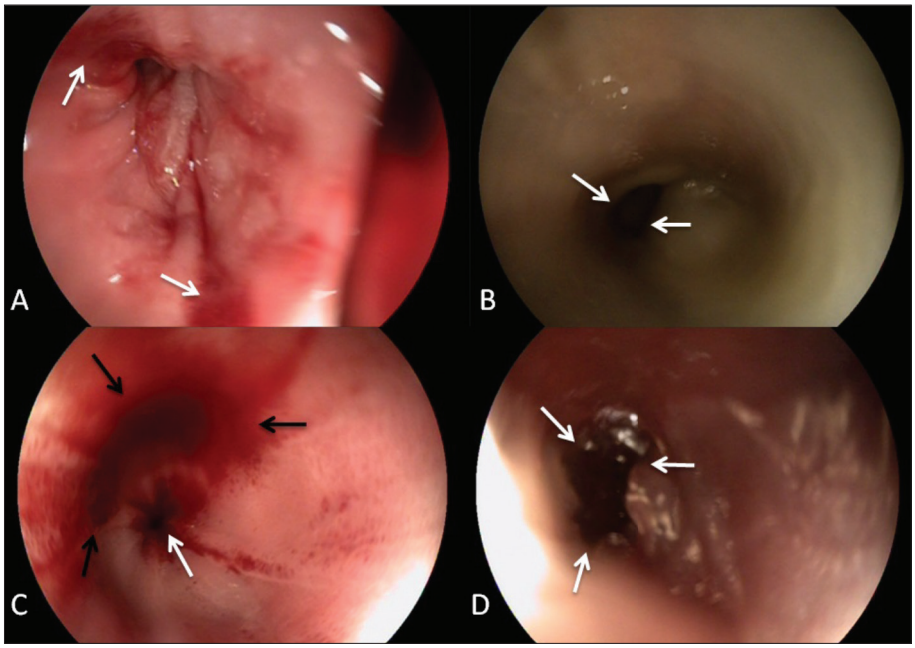


Fig 2. Examples from different atresia coli cases during colonoscopy: (A) Suspected type I atresia, with areas of mucosal erosions *(white arrows)* due to previous catheterization (B) colon lumen tapering to a narrow point *(white arrows)* (C) colon lumen coming to an abrupt end except a tiny orifice *(white arrow),* the erythematous area around the orifice *(black arrows)* was iatrogenic and caused by the endoscope (D) mucosal wall of the blind end with a dark, reddish-brown necrotic area *(white arrows)*

The breed, gender, time elapsed after birth, atresia type, mucosa condition and colonoscopy duration of each case was shown on *Table 1*.

DISCUSSION

The condition mostly occurs in Holstein-Friesians because of a homozygous recessive inheritance factor and is sporadically seen in other breeds [4]. In our study, most of the effected calves were Simmental; this is most likely due to the large number of Simmental breeders in the region of our hospital.

Despite being one of the most common malformations seen in calves, there is no consensus on the etiology or surgical treatment of atresia coli [4]. Surgical correction by means of anastomosis or colostomy has been suggested even though the survival rate is rather low and post-operative care is demanding [4]. There are valid points on not correcting the condition as it is not extensively known to be non-hereditary in most breeds so there is a chance of keeping the calf with defective hereditary traits in the herd. Also, considering the low survival rate and operation costs, the owner is taking a financial risk if the surgery is done. However, there are reports on having healthy calves from surgically corrected animals [9] and many owners that come to our hospital look favourably on surgical correction if every advantage and disadvantage of the operation is explained to them.

The diagnosis of atresia coli is usually easy, meconium is not passed through after birth and there is an anal opening,

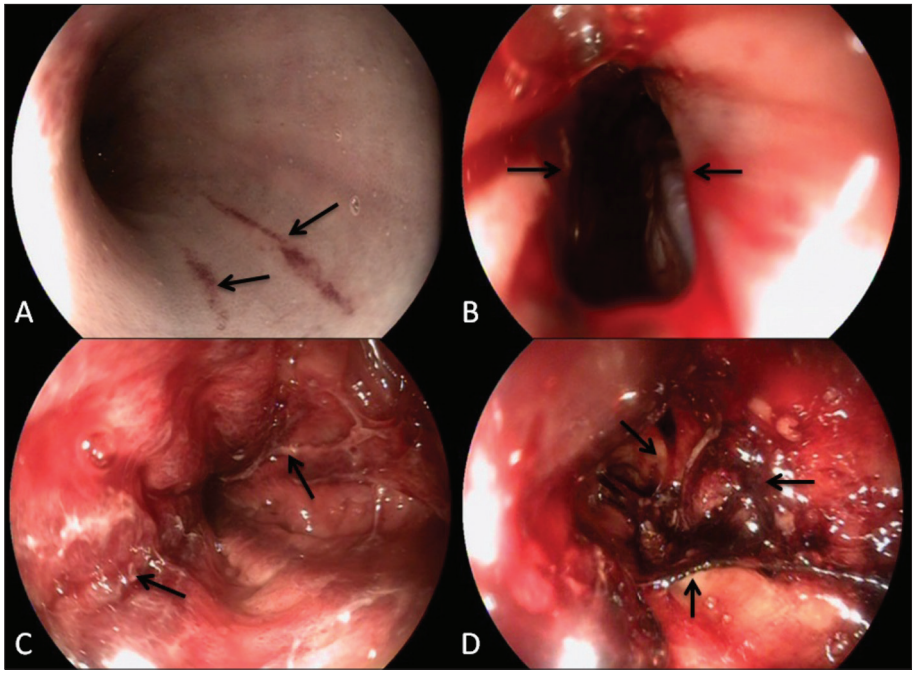


Fig 3. Examples of damage seen in the lumen during colonoscopy: Linear abrasions *(black arrows)* on the lumen (A), perforation *(between the black arrows, abdominal cavity can be seen)* on the wall of the blind end (B), mucosal adhesions *(black arrows)* surrounded by hyperemic mucosa (C), a large mass of clotted blood, mucous discharge and adhesions *(between the black arrows)* inside the lumen (D)

Table 1. *Age, breed, endoscopy duration and endoscopic findings of calves included in the study (Type 1-: Suspected type 1 atresia, Type 3-: Suspected type 3 atresia, Type 3: type 3 atresia)*

Case	Age (Days)	Breed	Gender	Colonoscopy Duration	Colonoscopy Reach (cm)	Atresia Type	Mucosa Damage
1	2	Holstein-Mix	Male	05:31	37	Type 1 -	-
2	2	Holstein-Mix	Male	06:02	40	Type 1 -	Linear abrasions
3	1	Simmental	Male	03:24	30	Type 3 -	Erythematous areas on the mucosa of blind end due to colonoscopy
4	3	Simmental	Male	04:25	30	Type 1 -	-
5	2	Simmental	Female	05:56	90	Type 1 -	-
6	6	Simmental	Female	04:32	30	Type 1 -	Diffuse clot covered areas lined with hyperaemic mucosa
7	3	Simmental	Female	07:57	22	Type 3	Linear superficial abrasions
8	4	Simmental	Male	06:41	45	Type 1 -	Linear superficial abrasions and bleeding due to colonoscopy
9	2	Simmental	Female	02:02	14	Type 3	Devitalized mucosa, adhesions, the blind end was perforated
10	4	Jersey	Male	06:43	25	Type 3 -	Abrasions and bleeding on the mucosa of blind end due to colonoscopy
11	4	Simmental	Male	04:06	60	Type 1	Superficial abrasions
12	7	Holstein-Mix	Male	03:20	25	Type 1	Clotted blood present in the lumen, hyperaemic and swollen mucosa
13	15	Holstein	Female	05:33	75	Type 1	Superficial, linear abrasions
14	13	Holstein-Mix	Male	02:30	45	Type 3	Swollen colon mucosa with diffuse erythematous areas, dark brown necrotic tissue on blind end
15	5	Simmental	Female	07:13	60	Type 3 -	Linear superficial abrasions due to colonoscopy
16	6	Simmental	Female	05:54	50	Type 1	Hyperaemic and slightly swollen areas only at the first 5 cm, rest of the colon was intact
17	2	Simmental	Female	02:53	20	Type 1	Diffuse abrasions that were bleeding
18	6	Limousin	Female	01:49	25	Type 1	Diffusely hyperaemic lumen
19	4	Simmental	Male	06:31	35	Type 3 -	Intraluminal adhesions, hyperaemic mucosa, blood clots

clinicians should suspect intestinal atresia. The condition is not immediately fatal so there is often enough time to get the animal to a reference hospital for diagnosis and treatment [12]. In this study, some of the cases were brought in very late, suggesting the owners mistook the mucus coming from the anal opening for meconium. Furthermore, most of the cases that were brought (n=11) had already damaged colon mucosae with one blind

end confirmed to be perforated, meaning the owners or practitioners tried to insert a catheter inside the atretic colon, which has a very fragile mucosal lining. In three cases, the mucosal damage was extensive and the mucosa had necrotic areas due to the passage of time after being traumatized. While the number of cases presented in this study may not reflect a larger group, they still suggest the fact that many calves will be brought to the hospital with traumatized mucosal linings. It is possible to see the blind end of the colon with direct radiography and ultrasonography [12,13], and one study shows that retrograde contrast radiography can reasonably show the atretic segment [11]. Exploratory laparotomy is the definitive way to establish diagnosis and determine the atresia type [12]. None of these techniques provide a way to noninvasively evaluate the colon mucosa, although one study investigated potential damage to the intestines through the use of biomarkers [14]. Atresia coli surgery involves either performing a colostomy or an anastomosis of the intestines, and even after surgery, functional obstruction may block the flow of feces [1]. Performing colonoscopy not only aids in diagnosis but during the surgical planning process, as seeing a necrotic, infected or obliterated colon segment during the surgery may call for euthanasia and prevent wasting valuable time and resources. Also it helps the surgeon decide in choosing colostomy over anastomosis if the colon segment attached to the rectum is too short, too stenotic or badly damaged. Colonoscopy was previously performed to diagnose the atresia coli in a foal with success [15], which shows the technique may be used for diagnosis, and the results of this study show that it can be used for diagnosis in calves.

Colonoscopy requires minimal experience, is cheap to perform, and does not require sedation or anaesthesia. Familiarizing with the technique requires only a few tries and it can be done in around 5 min. We believe colonoscopy should be used for diagnosis in atresia coli cases and is quite useful for pre-operative planning, aids in determining prognosis and explaining the condition to the owners. The only drawbacks are that endoscopy is not readily available in every clinic and its use is usually limited to reference hospitals and iatrogenic damage to the mucosa is possible during its use.

REFERENCES

1. Kilic N, Sarierler M: Congenital intestinal atresia in calves: 61 cases. *Rev Med Vet,* 155 (7): 381-384, 2004.

2. Van der Gaag I, Tibboel D: Intestinal atresia and stenosis in animals: A report of 34 cases. *Vet Pathol,* 17 (5): 565-574, 1980. DOI: 10.1177/030098588001700505

3. Johnson R: Intestinal atresia and stenosis: A review comparing its etiopathogenesis. *Vet Res Commun,* 10 (1): 95-104, 1986. DOI: 10.1007/BF02213972

4. Constable PD, Shanks RD, Huhn J, Morin DE: Evaluation of breed as a risk factor for atresia coli in cattle. *Theriogenology,* 48 (5): 775-790, 1997. DOI: 10.1016/S0093-691X(97)00301-4

5. Romano JE, Bryan K, Ramos RS, Velez J, Pinedo P: Effect of early pregnancy diagnosis by per rectum amniotic sac palpation on pregnancy loss, calving rates, and abnormalities in newborn dairy calves. *Theriogenology,* 85 (3): 419-427, 2016. DOI: 10.1016/j.theriogenology.2015.09.004

6. Romano JE, Pinedo P, Bryan K, Ramos RS, Solano KG, Merchan D, Velez J: Comparison between allantochorion membrane and amniotic sac detection by per rectal palpation for pregnancy diagnosis on pregnancy loss, calving rates, and abnormalities in newborn calves. *Theriogenology,* 90, 219-227, 2017. DOI: 10.1016/j.theriogenology.2016.11.004

7. Syed M, Shanks R: Atresia coli inherited in holstein cattle. *J Dairy Sci,* 75 (4): 1105-1111, 1992. DOI: 10.3168/jds.S0022-0302(92)77855-2

8. Kumar PR, Prasad VD, Sreenu M, Krishna N: Surgical management of atresia ani in calves-comparative evaluation of timely and delayed presentation complications. *Intas Polivet,* 18 (2): 386-389, 2017.

9. Smith DF, Ducharme NG, Fubini SL, Donawick WJ, Erb HN: Clinical management and surgical repair of atresia coli in calves: 66 cases (1977-1988). *J Am Vet Med Assoc,* 199 (9): 1185-1190, 1991.

10. Azizi S, Mohammadi R, Mohammadpour I: Surgical repair and management of congenital intestinal atresia in 68 calves. *Vet Surg,* 39 (1): 115-120, 2010. DOI: 10.1111/j.1532-950X.2009.00611.x

11. Abouelnasr K, Ishii M, Inokuma H, Kobayashi Y, Lee K, Yamada K: Atresia coli in a Japanese black calf diagnosed by a barium sulphate enema contrast radiograph in the standing position: A case report. *Vet Med (Praha),* 57 (7): 376-379, 2012. DOI: 10.17221/6267-VETMED

12. Trent A, Ducharme N, Fubini S, Steiner A: Surgery of the Calf Gastrointestinal System. **In,** Fubini SL, Ducharme N (Eds): Farm Animal Surgery. 2nd ed., 505-518, Elsevier Health Sciences, St. Louis, 2017.

13. Ducharme NG, Arighi M, Horney FD, Barker IK, Livesey MA, Hurtig MH, Johnson RP: Colonic atresia in cattle: A prospective study of 43 cases. *Can Vet J,* 29 (10): 818-824, 1988.

14. Yildiz R, Ok M, Ider M, Aydogdu U, Naseri A, Parlak K, Gulersoy E: Evaluation of intestinal damage biomarkers in calves with atresia coli. *J Vet Res,* 62 (3): 379-384, 2018. DOI: 10.2478/jvetres-2018-0054

15. Hunter B, Belgrave RL: Atresia coli in a foal: Diagnosis made with colonoscopy aided by N-butylscopolammonium bromide. *Equine Vet Educ,* 22 (9): 429-433, 2010. DOI: 10.1111/j.2042-3292.2010.00080.x

Kafkas Univ Vet Fak Derg
26 (2): 171-180, 2020
DOI: 10.9775/kvfd.2019.22647

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Effects of Form of Dietary Trace Mineral Premix on Fertility and Hatchability of Broiler Breeder Hens and Post-Hatch Performance and Carcass Parameters of Their Progenies

Seyyednaeim SABER [1,a] Hasan Rustu KUTLU [1,b] Yusuf UZUN [1,c]
Ladine CELIK [1,d] Ozcan YUCELT [1,e] Mikail BAYLAN [1,f]

[1] Çukurova University, Faculty of Agriculture, Department of Animal Science, TR-01330 Adana - TURKEY
[a] ORCID: 0000-0002-9635-7105; [b] ORCID: 0000-0002-3891-1534; [c] ORCID: 0000-0002-0493-7787; [d] ORCID: 0000-0003-3352-9181
[e] ORCID: 0000-0002-7371-5440; [f] ORCID: 0000-0002-6299-5811

Article ID: KVFD-2019-22647 Received: 16.05.2019 Accepted: 21.09.2019 Published Online: 21.09.2019

How to Cite This Article

Saber S, Kutlu HR, Uzun Y, Celik L, Yucelt O, Baylan M: Effects of form of dietary trace mineral premix on fertility and hatchability of broiler breeder hens and post-hatch performance and carcass parameters of their progenies. *Kafkas Univ Vet Fak Derg,* 26 (2): 171-180, 2020.
DOI: 10.9775/kvfd.2019.22647

Abstract

The present study was conducted to investigate the effects of dietary inorganic and/or organic based trace mineral premix in full or half doses in broiler breeders' diet on hatchability, fertility, post hatch performance, and carcass parameters of broiler progeny. A total of 220 Ross-308, 36 wk-old broiler breeders were divided into 4 dietary treatments (100% inorganic, 50% organic + 50% inorganic, 50% organic, and 100% organic trace mineral premix) with 5 replicates of 10 females and 1 male in each pen having similar body weight and egg production. The results of this experiment showed that inclusion of trace minerals source type in broiler breeders' diet did not have a significant effect on hatching egg characteristic. The results, however, showed that diets containing full (100%) or half (50%) doses of organic and/or inorganic minerals in broiler breeder hens' diet have a significant effect on fertility rate ($P<0.05$). The results also showed that growth performance of offspring was, however, not affected by the trace mineral sources or level used in the maternal diets at the end, while carcass weight and carcass yield were significantly affected. It may be concluded that replacing inorganic based trace mineral premix with half or full dose of organic based trace mineral premix in the broiler breeder hens' diet could improve hatching performance, growth and carcass performances of their progenies.

Keywords: *Broiler breeder hen, Reproduction, Hatchability, Trace minerals, Progeny*

Broyler Dişi Damızlık Rasyonlarında Kullanılan İz Mineral Premiksi Formunun Üreme Performansı, Kuluçka Özellikleri ve Kuluçka Sonrası Civcivlerde Büyüme Performansı ve Karkas Parametreleri Üzerine Etkileri

Öz

Mevcut çalışma, rasyon bileşiminde kullanılan organik ve inorganik iz mineral premikslerinin broyler damızlıklarda döllülük oranı, kuluçka randımanı ve kuluçka sonrası civcivlerde büyüme performansı ve karkas parametreleri üzerine etkisini belirlemek amacıyla yürütülmüştür. Canlı ağırlık, yumurta verimleri benzer olan 36 haftalık yaşta toplam 220 adet Ross-308 broyler damızlık tavuk 4 muamele grubuna %100 inorganik, %50 organik + %50 inorganik, %50 organik ve %100 organik iz mineral premiks) her bir grup, her birinde 10 dişi ve 1 erkek bulunan 5 alt gruptan oluşturulmuştur. Rasyonda organik ve/veya inorganik premikslerin broyler damızlıkların beslenmesinde kullanımının kuluçkalık yumurta kalitesi üzerine önemli etkiye sahip olduğu saptanmıştır. Öte yandan, damızlık tavukların rasyonlarında %100 veya %50 oranında organik ve inorganik kaynaklı minerallerin kullanımının döllülük oranı üzerine olan etkisi önemli ($P<0.05$) bulunmuştur. Denemeden elde edilen sonuçlara göre dişi ebeveyinleri organik ve/veya inorganik iz mineral kaynakları ile beslenen civcivlerin performanslarının benzer; ancak rasyon organik iz mineral kaynağının civcivlerin karkas ağırlığı ve karkas randımanı üzerinde etkisinin olumlu olduğu saptanmıştır. Sonuç olarak broyler damızlık tavuk rasyonlarında inorganik iz mineral yerine %100 veya %50 organik iz mineral premiksi kullanımının broyler damızlıklarda kuluçka randımanı ve civcivlerinde büyüme performansı ve karkas verimini iyileştirdiği gözlenmiştir.

Anahtar sözcükler: *Broyler dişi damızlık, Üreme, Kuluçka Randımanı, İz Mineral, Civciv*

INTRODUCTION

Producing fertile eggs for gaining the highest hatchability is one of the important needs of having profitable chicken breeding. In poultry, many factors such as parents and environment may influence on production of egg characteristics [1] and its fertility [2]. In poultry, diets trace minerals are essential as they play important roles on

İletişim (Correspondence)
+90 531 3296266
naeim_saber@yahoo.com

male and female breeders' reproduction performance, biochemical processes required and embryo development [3-5]. It has been reported that good nutritional condition of the parents is vital for transferring of required nutrients for development of the embryo [6,7]. Some important interrelationships between several micronutrients and other nutrients have been reported and these interrelationships may affect the reproduction and production of hens, especially during the last period of production [8]. Zinc is one of the most important essential trace minerals for the growth, immune system function, reproduction, biochemical processes, and also is a cofactor for many metalloenzymes. During embryogenesis, zinc is reported to be a very important nutrient for embryo as utilizing of zinc during 11th to 17th days decreases from 0.99 mg to 0.01 [9]. Source of this mineral could also be an important factor as the inclusion of organic zinc (80 ppm zinc-methionine) in broiler breeders' diet improves cellular immune response [10]. It was reported that Cu has an influence on 17 beta-estradiol and enzymes involved in carbohydrate, lipid, and amino acid metabolism in mature laying hens [11]. Replacements of 30% inorganic sources of Zn, Mn, and Cu with organic sources of this micro-element in broiler breeders' diet have been reported to improve humoral immune responses [12] and gastro intestinal development of their offspring's [13]. It has also been reported that supplementation of broiler breeder hen's diets with simultaneous inorganic and organic forms of Zn, Mn, and Cu had significant effects on egg shell quality, embryo mortality, some bone mineralization parameters, tibia calcification, and thickness [14,15]. Iron (Fe) is also known an essential trace mineral for all living organisms, and it is required for several metabolic processes, including oxygen and electron transport as well as DNA synthesis [16]. Selenium is normally provided in the diet in the form of inorganic sodium selenite. An organic form can be provided, which is selenium-enriched yeast. Yeast, like plants, form selenoamino acids and other organic selenocompounds that exist in very reduced form in comparison to the highly oxidized inorganic selenium forms (selenite and selenate). The protective effects of organic selenium are reported to be especially apparent during the highly oxidative state of late incubation and the first few days after hatch [17,18]. Although some advantages have been obtained in recent years, inclusion of organic-based trace minerals in poultry diets could not be a common practice as they are expensive. It has been speculated that as the digestibility and bio-availability of organic based trace minerals are high, half dose of inorganic sources could be used in the premixes and then the cost could be reduced. The present experiment was conducted to determine if dietary inorganic vs. organic based trace mineral premix used in half (50%) or full (100%) or doses would affect fertility and hatching performance of broiler breeder hens and post-hatch performance and carcass parameters of their progenies.

MATERIAL and METHODS

The present study was carried out in the Broiler Unit of Experimental Farm of the Department of Animal Science, Faculty of Agriculture, University of Cukurova-Turkey. All the protocols used in this experiment were approved by the Animal Experiments Local Ethics Committee of Cukurova University, Adana-Turkey.

General Experimental Procedure and Trial Groups

In this experiment, two hundred and twenty, aging 36 weeks Ross-308 broiler breeders (200 female + 20 male) were used for 10 weeks. During pre-feeding period (36th to 39th weeks), all broiler breeder hens *(Table 1)* or cocks *(Table 2)* were fed with standard breeder hen's or cock's diets based on corn and soybean which were formulated according to the recommendation of Ross Breeding Company [19].

All birds after pre-feeding period were divided into four treatment groups according to mean body weight and mean egg production in a complete randomized design. The treatment groups were differ in dietary trace mineral premixes (A: 100% inorganic, B: 50% organic + 50% inorganic, C: 50% organic, and D: 100% organic) with five replicate pens. The contents of organic and inorganic trace minerals in premixes were shown in *Table 3*.

The birds were accomodated in 20 pens, each (2×1.5×2 m) included ten females and one male, wood shaving litter (7-8 cm height), female tubular feeder, one individual male feeder, and nipple drinkers. A 16:8 light:dark photoperiod program was applied and feeds were given to breeders (female: 163 g/day, male: 130 g/day) according to the recommendation of Ross Breeding Company [19] with ad libitum access to drinking water. During the experiment, environmental temperature (18-22°C) and humidity (55-60% RH) were maintained within the animal comfort zone using foggers and tunnel ventilation. Throughout the experiment, males' mating performances were watched every day. The male with low mating performance was replaced with a spare one.

Fertility and Hatching Performance

At the 43th week of age, eggs were checked and collected for fertility test (with bright light) to understand male broiler breeders reproduction performance before incubation. Hatching performance of the eggs obtained from the birds was examined at the end of 21 day incubation. For this purpose, at 44th and 45th weeks of age all eggs were selected (removing double yolk eggs, dirty eggs, thin shell eggs, broke eggs, and crack eggs) and stored in a cool room (12°C with 70% RH) until obtaining 700 eggs (35 for each replicate) The eggs were incubated in a single stage incubator (Cimuka Inc., Ankara, Turkey) at 37.2°C and a 10 RH of 55%) according to McQuoid [20]. At the end of 21st day

Table 1. *Ingredient and nutrient compositions of the experimental diets given broiler breeder hens*

Ingredients (%)	Trace Mineral Source of Experimental Diets			
	100% Inorganic	50% Organic + 50% Inorganic	50% Organic	100% Organic
Yellow corn	54.49			
Soybean meal (47.5% CP)	10.00			
Full fat soybean	9.64			
Sunflower meal (36% CP)	7.46			
Corn gluten meal (60% CP)	3.86			
Meat-bone meal (35%CP)	2.48			
DCP (18% P)	1.57			
Soybean oil	2.00			
Limestone	7.61			
Common salt	0.24			
Sodium bicarbonate	0.10			
L-Lysine	0.06			
Choline-60	0.05			
DL-methionine	0.04			
Vitamin premix[1]	0.20			
Trace mineral premix[2]	0.20 Inorganic	0.10 Inorganic + 0.10 Organic	0.10 Organic	0.20 Organic
Limestone	-	-	0.10	-
Total	100.00	100.00	100.00	100.00
Nutrients (%)				
Dry matter	88.52			
Crude protein	19.00			
Crude fiber	3.58			
Ether extract	3.71			
Ash	13.35			
Starch	34.99			
Lysine	0.87			
Methionine	0.37			
Methionine + Cystein	0.70			
Tryptophane	0.20			
Thronine	0.70			
Ca	3.64			
Available phosphorus	0.50			
Sodium	0.16			
Metabolizable energy (kcal/kg)	2680			

[1] Vitamin premix (per 2 kg of diets) 15.000.000 IU Vit. A, 5.000.000 IU Vit. D_3, 100.000 mg Vit. E, 3.000 mg Vit. K_3, 3.000 mg Vit. B_1, 8.000 mg Vit. B_2, 60.000 mg Niasin, 15.000 mg Ca-D-Pantotenat, 5.000 mg Vit. B_6, 20 mg Vit. B_{12}, 2.000 mg Folic Acid, 200 mg D-Biotin, 100.000 mg Vit. C; [2] see Table 3

Table 2. *Ingredient and nutrient compositions of the experimental diets given broiler breeder cocks*

Ingredients	(%)	Nutrients (%)	
Yellow corn	62.44	Dry matter	86.89
Wheat middling	23.76	Crude protein	12
Sunflower meal-36	10.79	Crude fiber	5.96
Limestone (Grn)	1.18	Ether extract	3.30
DCP-18	0.70	Ash	4.80
Sodium bicarbonate	0.22	Starch	45.58
Salt	0.22	Ca	0.70
Carboxylic acid (Salkil)	0.20	TOT-P	0.63
L-Lysine	0.13	Na	0.16
Vitamin premix[1]	0.10	Metabolizable Energy (kcal/kg)	2750
Mineral premix[2]	0.10		
DL-methionine	0.06		
Choline-60	0.05		
Availa Se 1000 (Zinc-L-selenomethionine)	0.05		
Total	100		

[1] Vitamin premix (per 2 kg of diet): Vit. A, 16.000 IU; Vit. D_3, 3.000 IU; Vit. E, 40 IU; Vit. K_3, 2.5 mg; Vit. B_1, 2.5 mg; Vit. B_2, 10 mg; Nicotinamide, 50 mg; Calcium D-pantothenate, 15 mg; Vit. B_6, 6.25 mg; Vit. B_{12}, 0.035 mg; Folic acid, 15 mg; D-biotin, 0.045 mg; Choline chloride, 150 mg; [2] Mineral premix (mg/kg of diet): Mn, 80; Fe, 80; Zn, 60; Cu, 8; Co, 0.2; I, 0.5; Se, 0.15

incubation, all hatched broilers were carefully taken from the pouches and weighted by a digital scale with 0.01g sensitivity. After weighting, the chicks were sexed by wing feathers according to Ross [21] recommendation. Eggs that did not hatch were then broken to determine embryo diagnosis for classification of eggs as infertile or dead embryos. A visual estimation of the age at death stage was carefully performed and embryonic mortality was separated as early (1 to 7 days), intermediate (8 to 14 days) or late (15 to 21 days) dead in shell [22]. The percentage of hatching chicks considered improper for placement as well as pips were calculated. The difference between total eggs set and infertile eggs allowed the calculation of present hatchability of fertile eggs. So, fertility rate (%), total hatchability (%), and hatchability of fertile eggs (%) were calculated [23] as given below;

Fertility rate (%) = (No of fertile eggs/No of eggs placed in hatchery) ×100

Total hatchability (%) = (No of chicks hatched/No of eggs placed in hatchery) × 100

Hatchability of fertile eggs (%) = (No of chicks hatched/No of fertile eggs placed in hatchery) × 100

Growth Performance of Progeny

At the end of the incubation, all chicks obtained from the eggs were carefully taken and divided into 20 pens by name of the maternal group/subgroup number to follow on the same axis to maternal to its chicks. Each treatment group had five pens sized 2×3 m that were equipped with a tube feeder, an automatic water-bowl on litter, and wood shaving litter with 7-8 cm height. During the experiment, all chicks were fed with starter (0-10 days of age), grower

Table 3. *Sources and contents of trace mineral premixes in organic or inorganic forms used in the experiment*

Trace Mineral		Source	Source Amount in Mix	Trace Mineral Amount in Mix
Inorganic form (per 2 kg)	Manganese	$MnSO_4$ (32%)	250.000 mg	80.000 mg
	Iron	$FeSO_4$ (30%)	200.000 mg	60.000 mg
	Zinc	ZnO (72%)	83.333 mg	60.000 mg
	Copper	$CuSO_4$ (77%)	6.494 mg	5.000 mg
	Selenium	Na_2SeO_3 (4.5%)	4.444 mg	0.200mg
	Cobalt	$CoSO_4$ (20%)	1.000 mg	0.200 mg
	Iodine	$Ca(IO_3)_2$ (62%)	1.613 mg	1.000 mg
	Filling material	Limestone	1.453.116 mg	
	Total		2.000.000 mg	
Organic form (per 2 kg)	Manganese	Mintrex Mn (Metionin-Hid. Analog Mn Chelate 15.5%)	516.129 mg	80.000 mg
	Iron	Mintrex Fe (Methionine-Hid. Analog Fe Chelate 16.0%)	375.000 mg	60.000 mg
	Zinc	Mintrex Zn (Metionin-Hid. Analog Zn Chelate 17.5%)	342.857 mg	60.000 mg
	Copper	Mintrex Cu (Metionin-Hid. Analog Cu Chelate 18.0%)	27.777 mg	5.000 mg
	Selenium	ZORIEN SeY, 2% Se)	10 mg	0.200 mg
	Cobalt *	$CoSO_4$ (20%)	1.000 mg	0.200 mg
	Iodine *	$Ca(IO_3)_2$ (62%)	1.613 mg	1.000 mg
	Filling material	Limestone	735.614 mg	
	Total		2.000.000 mg	

** They have no organic form*

(11-21 days of age), finisher (22-35 days of age), and withdrawal (36-42 days of age) diets *(Table 4)* that they were formulated by Ross [21] recommendation.

During the experiment (42 days) all chicks were fed ad libitum under 23:1 light: dark photoperiod. Environmental temperature in animal house was controlled by heating and tunnel ventilation system starting from 33°C in the first week and gradually decreased by 3°C per week until the fourth week then it fluctuated between 22-24°C. During the experiment, live weight, feed intake, and feed efficiency were recorded weekly.

Carcass Parameters of Progeny

In order to determine the carcass weight, abdominal fat and carcass yield, at 42nd day of age all chicks were weighted and 6 chicks (3 males + 3 females) from each subgroup were selected according to the average body weight and then slaughtered.

Statistical Analysis

The data obtained in the study were analyzed using GLM (General Linear Model) procedure of the Statistical Analysis System [24] to obtain the effect of trace mineral source. Duncan's New Multiple Range Test in SAS was used to identify significant differences among treatments means [24]. Results obtained in this study were presented as means per bird with standard errors of the difference between means [SED; √(S^2/n)] with P values, except for feed intake of breeders as feeds were given to the them in equal amounts according to the recommendation of the Breeding Company [19].

RESULTS

Effects of dietary trace mineral sources on number of hatchable eggs in broiler breeders were given in *Table 5*. The results showed that source or dose of dietary trace minerals did not have any significant effects on number of hatchable eggs (P>0.05). But use of organic based trace minerals in broiler breeder hens' diet numerically increased number of hatchable eggs.

Results related fertility and hatching performance of broiler breeder hens were given in *Table 6*.

The data obtained from this experiment revealed that the inclusion of trace minerals in broiler breeders' diet have significant effects on some hatching performance such as number of fertile eggs, fertility rate, and offspring (female) livability (P<0.05). But inclusion of organic and inorganic trace minerals did have no significant effects on embryonic mortality, number of chicks hatched alive, chicks' weight at hatching, post-hatch mortality, hatchability of fertile eggs, total hatchability, and offspring (male) livability (P>0.05). In broiler breeders, some of hatching performance such as embryonic mortality in late stage, number of chicks hatched alive, hatchability of fertile eggs, post-hatch mortality, total hatchability and offspring (male) livability

Table 4. *Ingredient and nutritional compositions of broiler chicks' diets*

Ingredients (%)	Starter (0-10 d)	Grower (11-21 d)	Finisher (22-35 d)	Withdrawal (36-42 d)
Yellow corn	43.17	46.63	50.70	50.76
Soybean meal (47.5% CP)	15.64	7.71	-	-
Full fat soya	14.17	16.68	26.21	26.21
Wheat short (15% CP)	13.03	13.00	11.17	11.17
Maize gluten meal (60% CP)	5.00	3.00	-	-
Poultry offal meal (52% CP)	-	4.00	4.00	4.00
Meat-bone meal (33% CP)	4.00	5.27	4.49	4.49
Soya oil	2.00	2.00	2.00	2.00
DCP (18% P)	0.60	-	-	-
Sodium bicarbonate	0.11	0.08	-	-
Common salt	0.17	0.14	0.21	0.21
Bio-lysine (60%)	0.77	0.60	0.36	0.36
Limestone	0.61	0.28	0.26	0.26
DL-methionine	0.36	0.25	0.24	0.24
Anticoccidial	0.06	0.06	0.06	-
Vitamin Premix*	0.20	0.20	0.20	0.20
Mineral Premix**	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00
Nutrients (%)				
Dry matter	88.00	88.00	88.00	88.00
Crude protein	24.00	22.00	21.00	20.00
Ether extract	7.00	8.66	10.13	10.13
Crude fiber	3.20	3.17	3.37	3.37
Crude ash	6.03	5.80	5.48	5.48
Lysine	1.43	1.26	1.09	1.09
Methionine	0.70	0.56	0.50	0.50
Methionine + Cystine	1.07	0.84	0.86	0.86
Calcium	1.00	1.00	0.90	0.90
Available phosphorus	0.45	0.45	0.40	0.40
Sodium	0.16	0.16	0.16	0.16
Metabolizable energy (kcal/kg)	3050	3150	3250	3250

** Each 2 kg of vitamin premix contains 15.500.000 IU Vit. A, 5.000.000 IU Vit. D_3, 100.000 mg Vit. E, 3.000 mg Vit. K_3, 3.000 mg Vit. B_1, 8.000 mg Vit. B_2, 60.000 mg Niacin, 15.000 mg Ca-D-Pantotenate, 5.000 mg Vit. B_6, 20 mg Vit. B_{12}, 2.000 mg Folic acid, 200 mg D-biotin, 100.000 mg Vit. C; ** Each kg of inorganic trace mineral premix contains 80.000 mg Mn, 60.000 mg Fe, 60.000 mg Zn, 5.000 mg Cu, 200 mg Co, 1000 mg I, 200 mg Se (sodium selenite), 500.000 Choline chloride*

were numerically improved in the groups received diets containing organic based trace minerals. According to *Table 5*, the number of fertile eggs, fertility rate, total hatchability, hatchability of fertile eggs, and livability of broiler seemed to be higher in groups which received diets containing organic-based trace minerals than the groups which received 100% inorganic-based trace minerals.

Table 7 summarizes the impacts of dietary organic and inorganic trace minerals used in maternal diet on progeny performance.

The results indicated that source of trace mineral used in maternal diet did not have a significant effect on feed intake, feed conversion ratio, male livability, and male body mass of offspring (P>0.05). However, it was seen that source or amount of trace minerals in their female parents' diets had a significant effect on progenies' body weight at 21th days of age (P<0.05), while it was disappeared at the 42th days of age. The results also showed that inclusion of organic and inorganic trace minerals in broiler breeders' diet have significant effects on female livability, female body mass, and average body mass in offspring (P<0.05).

Table 8 illustrates the data obtained from carcass parameters of offspring. The results indicated that the inclusion of

Table 5. *The effect of maternal dietary trace minerals on hatching egg characteristic (Number/group/2 weeks)*

Parameters	Groups				SED	P
	100% Inorganic	50% Organic + 50% Inorganic	50% Organic	100% Organic		
No of total egg	82.0	85.2	79.6	87.4	2.88	0.784
No of hatchable eggs	70.6	75.6	67.6	75.4	2.32	0.563
No of un-hatchable eggs	11.40	9.60	12.00	12.00	1.02	1.818
Double yolk eggs	-	-	-	-	-	-
Dirty eggs	5.0	5.2	5.8	5.4	0.81	0.987
Thin shell eggs	0.2	0.0	0.0	0.0	0.05	0.418
Broke eggs	1.0	0.8	0.8	1.0	0.17	0.952
Crack eggs	5.0	3.6	5.6	5.6	0.78	0.777

Table 6. *Effect of maternal dietary trace minerals on hatching performance in broiler breeders*

Parameters	Groups				SED	P
	100% Inorganic	50% Organic + 50% Inorganic	50% Organic	100% Organic		
Number of eggs placed hatchery (no/replication)	39	39	39	39	-	-
Number of fertile eggs (number/replication)	33.00^{b}	37.00^{a}	34.60^{ab}	36.00^{ab}	0.48	0.058
Embryonic mortality in early stage	1.66	1.33	1.00	2.00	0.19	0.731
Embryonic mortality in mid stage	-	-	-	-		-
Embryonic mortality in late stage	3.50	3.00	2.20	2.33	0.32	0.509
Number of chicks hatched alive	26.00	31.00	30.20	30.75	1.31	0.542
Male (number)	11.75	15.40	15.80	15.25	0.94	0.452
Female (number)	14.25	15.60	14.40	15.50	0.79	0.892
Chicks' weight at hatching (g/chick)	48.71	48.15	47.50	46.91	0.29	0.174
Post-hatch mortality (number)	2.66	2.20	2.00	2.25	0.38	0.962
Fertility rate (%)	84.62^{b}	94.87^{a}	88.71^{ab}	92.30^{ab}	1.24	0.058
Hatchability of fertile eggs (%)	78.21	83.42	87.03	85.17	2.97	0.763
Total hatchability (%)	66.66	79.48	77.43	78.84	3.35	0.542
Offspring - livability - male (%)	82.69	95.00	84.17	100.00	3.00	0.181
Offspring - livability female (%)	96.87^{a}	95.24^{a}	85.41^{b}	96.65^{a}	1.54	0.051

^{a,b} *Means within a row lacking a common superscript differ significantly (P<0.05)*

different forms and levels of trace minerals in maternal' diet had a significant effect on offspring carcass weight, and carcass yield (P<0.05) without a significant effect on abdominal fat (P>0.05).

DISCUSSION

It is well known that fulfilment of dietary trace mineral needs of poultry is very important to maintain production and product quality. Trace minerals such as iron, manganese, zinc, copper and selenium play many significant roles as enzyme cofactors and as constituents of metalloenzymes, either individually or in combination, in supporting growth, production and maintenance of the structural integrity of tissues. As the efficacy of the use of microelements is an important issue in modern poultry nutrition, various studies on sources and their bioavailability of trace minerals have been under investigation [25]. The availability of minerals from feed materials of plant origin, as well as from traditional inorganic sources, *i.e.*, oxides, sulphates, or carbonates, is relatively low, while the requirements of modern, high-producing lines of laying hens and broiler chickens for microelements are very high. These facts, along with advanced knowledge of the importance of micro-elements in immunological processes and reproduction and the variable content of trace minerals in feed materials, has lead, in commercial practice, to their being added to poultry diets in high amounts, with a large safety margin, often exceeding the birds' requirements [26], leading to soil and water pollution through excrete. In recent years, use of organic based trace minerals in poultry could be of value in terms of fulfilment of trace mineral needs of farm

Table 7. *The effect of maternal dietary trace minerals on growth performance of offspring*

Day	Parameters	Groups				SED	P
		100% Inorganic	50% Organic + 50% Inorganic	50% Organic	100% Organic		
7	Feed intake (g/chicks)	190.9	166.40	192.88	160.91	12.22	0.717
	Body weight (g/chicks)	96.54	111.52	102.79	108.10	2.53	0.213
	Feed Conversion Rate	1.99	1.52	1.87	1.49	0.12	0.414
14	Feed intake (g/chicks)	508.4	506.52	515.33	518.95	11.98	0.980
	Body weight (g/chicks)	383.3	407.93	398.03	404.97	5.17	0.367
	Feed Conversion Rate	1.32	1.24	1.28	1.28	0.02	0.684
21	Feed intake (g/chicks)	1193	1187	1230	1291	31.76	0.645
	Body weight (g/chicks)	789.1^{b}	831.7^{ab}	837.2^{ab}	866.2^{a}	8.23	0.032
	Feed Conversion Rate	1.51	1.42	1.46	1.49	0.03	0.796
28	Feed intake (g/chicks)	2223	2254	2287	2362	49.66	0.781
	Body weight (g/chicks)	1402	1332	1315	1371	21.24	0.482
	Feed Conversion Rate	1.59	1.69	1.73	1.73	0.04	0.618
35	Feed intake (g/chicks)	3225	3444	3570	3569	63.19	0.214
	Body weight (g/chicks)	1940	1969	1973	2046	21.84	0.389
	Feed Conversion Rate	1.66	1.74	1.80	1.74	0.03	0.389
42	Feed intake (g/chicks)	4638	4757	4998	4974	69.78	0.238
	Body weight (g/chicks)	2614	2645	2709	2764	30.11	0.329
	Feed Conversion Rate	1.77	1.80	1.84	1.80	0.02	0.699

^{a,b} Means within a row lacking a common superscript differ significantly (P<0.05)

Table 8. *The effect of maternal dietary trace minerals on carcass parameters of offspring at 42 days old*

Parameters	Gender	Groups				SED	P
		100% Inorganic	50% Organic + 50% Inorganic	50% Organic	100% Organic		
Body weight (g/chicks)	Male	2949	2990	3051.	3001	17.07	0.218
	Female	2439^{b}	2428^{b}	2558^{a}	2559^{a*}	16.54	**0.004**
	Average	2645^{b}	2709^{ab}	2804^{a}	2780^{b}	18.01	**0.009**
Carcass weight (g/chicks)	Male	2107^{ab}	2078^{b}	2188^{a}	2109^{ab}	14.25	**0.046**
	Female	1735^{b}	1714^{b}	1817^{a}	1836^{a}	12.22	**0.009**
	Average	1886^{b}	1896^{b}	2003^{a}	1972^{a}	13.28	**0.004**
Abdominal fat (g/chicks)	Male	19.31	19.80	16.64	21.11	0.91	0.375
	Female	17.11	15.24	15.27	17.51	0.88	0.475
	Average	17.53	17.52	15.96	18.89	0.59	0.387
Abdominal fat (%)	Male	0.90	0.94	0.76	1.00	0.04	0.203
	Female	0.98	0.88	0.84	0.96	0.05	0.712
	Average	0.92	0.92	0.79	0.96	0.03	0.230
Carcass yield (%)	Male	71.39^{a}	69.524^{b}	71.71^{a}	70.32^{ab}	0.29	**0.035**
	Female	71.15	70.66	71.06	71.72	0.24	0.475
	Average	71.29^{a}	70.03^{b}	71.39^{a}	70.96^{ab}	0.02	0.067

^{a,b} Means within a row lacking a common superscript differ significantly (P<0.05)

animals while having less pollution and health problems as advised to use almost half as its high bioavalilibility. Recommendation for using organic based (chelated) trace minerals, containing a central metal atom (acceptor of electrons) together with ligands *(i.e.*, proteins, amino acids, carbohydrates, or lipids), at relatively low levels in

poultry diets has become widespread, especially due their ecological and physiological contributions; however there still is not enough experiment [27]. In fact, supplementing hens with highly bioavailable chelated sources of trace minerals would be expected to support not only the quality of eggs from breeder hens, but also hatching rate and progeny quality. The results obtained from the present study showed that replacing inorganic based trace mineral premix with half or full dose of organic based trace mineral premix in the broiler breeder hens' diet could improve hatching performance, growth and carcass performances of their progenies, as expected. The increments could be attributed to relatively high bioavilability and fulfilment capacity of animal needs for the trace minerals. Zinc is known to be a component of the carbonic anhydrase enzyme, which is crucial for supplying the carbonate ions during eggshell formation [28]; manganese is the metal activator of enzymes that are involved in the synthesis of mucopolysaccharides and glycoproteins that contribute to the formation of the organic matrix of the shell [29], and copper plays the role of cofactor of the lysyl-oxydase enzyme that is important in the formation of collagen cross links present in the egg shell membrane [30]. Not only zinc but also adequate Fe levels are also needed to maintain egg production and as well as hatching chicks' indexes. Taschetto et al.[31] reported that the average of dietary Fe requirement for broiler breeders' hen estimated to be about 100 ppm total. In the present study we provided 60 ppm supplemental Fe, but it is unknown what was the total Fe and other trace minerals in the diet, as no measurements for the trace elements examined in the present study were done for the diet before supplementation. Although we did not measure the contents of the trace minerals in the egg, our results with respect to growth and carcass performances of progenies suggest that organic based trace minerals had positive effects on, not only maternal, also progeny through eggs, as zinc is reported to be a very important nutrient for embryo as utilizing of zinc during 11^{th} to 17^{th} days decreases from 0.99 mg to 0.01 through embryonal development [9]. Sahin and Tasdemir [32] reported that 60 mg/kg organic based zinc (Zn-RedoxMin) supplementation instead of inorganic sources ($ZnSO_4$, ZnO and $ZnCl_2$) to breeder diets improved their chick quality and weight, but not feed conversion rate. Zamani et al.[33] reported that feeding layer hens with a diet containing different levels of manganese and zinc reduced number of crack-broken eggs while increasing egg production. The results of the present experiment revealed that replacing inorganic source with organic based source in half or full dose of trace mineral in broiler breeder hens' diets does not have a significant effect on hatching egg characteristics (P>0.05), however, some hatching performance parameters were affected by dietary trace mineral forms. The results obtained from this study were in line with Chen et al.[34] and Attia et al.[35] studies. Chen et al.[34] reported that inclusion of 250 mg copper ($CuSO_4$) in layer hens' diet increased concentration of copper in blood plasma and yolk, induced cholesterol content in yolk and blood plasma, and induced fertility rate but did not have any significant effect on total hatchability (P>0.05). On the other hand, it was reported that adding manganese, zinc, copper, and chromium in organic and inorganic form in layer breeders' diet did not have significant effects on laying performance and egg quality (P>0.05) but organic form of these minerals improved hatchability and hatching yield parameters [36]. In fact, it has been speculated that inorganic trace element does not fulfill trace element requirements of modern poultry due to their less bioavailability and negative interaction [37,38]. Organic based trace minerals could be of value as many reports have been published on their positive effects in layer, broiler and also turkey performance [14,15,39,40]. Previous studies also revealed that efficacy of organic based trace minerals in broilers could decrease as birds age increased [41,42]. However, no observations have been reported on efficacy of organic based trace minerals in breeders differing in age. Inclusion of inorganic (sulfate) and organic (amino acid chelate) forms on zinc in broiler breeders' diet improved reproduction performance while the immunity was positively affected by amino acid chelate form of zinc [43]. Inclusion of different forms of trace mineral in broiler breeders' diet does not have a significant effect on offspring receiving conventional (inorganic) trace mineral premix feed intake, feed conversion ratio (P>0.05), but there was a significant effect on female livability and average body mass (P<0.05). Rebel et al.[44] indicated that contents of lymphocyte resistance to various infections in offspring are increased due to feeding of their parents with a diet containing high levels of vitamin and minerals. The results with respect to positive effects of organic based trace elements on fertility and hatching performance of breeder hens and growth performance of progenies could be attributed to their higher bio-efficacy in their physiological roles from egg to hen. Sun et al.[45] reported that adding of organic form of Zn, Mn, and Se (Mintrex) instead of inorganic form of these microelements in broiler breeders' diet protected breeders from lipid peroxidation, increase their retention in the egg, and had a positive effect on growth performance of their offspring. Our results with respect to inclusion level of organic based trace minerals also supported the report of Aksu et al.[46], they concluded that organically complexed trace minerals can be used at a much lower concentration than the current recommended as inorganic based minerals, without a negative impact on performance, while also decreasing the excess mineral excretion.

In could be concluded that use of organic-based trace mineral (50% or 100%) in broiler breeder's diet improve number of total eggs and hatching eggs, fertility rate, total hatchability, hatchability of fertile eggs and also progeny livability. It would also be concluded that using organic-based trace mineral (50% or 100%) in broiler breeder's diet improve body mass and carcass yield of offspring.

Acknowledgements

We thank to TÜBITAK (project no: 114O749) for financial support. We also grateful to the Staff of Research and Application Farm of Agricultural Faculty, University of Cukurova for their valuable help and contribution.

REFERENCES

1. Wilson PB: Recent advances in avian egg science: A review. *Poult Sci,* 96, 3747-3754, 2017. DOI: 10.3382/ps/pex187

2. Fouad AM, Chen W, Ruan D, Wang S, Xia WG, Zheng CT: Impact of heat stress on meat, egg quality, immunity and fertility in poultry and nutritional factors that overcome these effects. *Int J Poultry Sci,* 15, 81-95, 2016. DOI: 10.3923/ijps.2016.81.95

3. Ruan H, Zhang Z, Wu Q, Yao H, Li J, Li S, Xu S: Selenium regulates gene expression of selenoprotein W in chicken skeletal muscle system. *Biol Trace Elem Res,* 45, 59-65, 2012. DOI: 10.1007/s12011-011-9166-y

4. Olgun O, Yazgan O, Cufadar Y: Effects of boron and copper dietary supplementation in laying hens on egg shell quality, plasma and tibia mineral concentrations and bone biomechanical properties. *Rev Méd Vét,* 163 (7): 335-342, 2012.

5. Juniper DT, Phipps RH, Bertin G: Effect of dietary supplementation with selenium-enriched yeast or sodium selenite on selenium tissue distribution and meat quality in commercial-line turkeys. *Animal,* 5, 1751-1760, 2011. DOI: 10.1017/S1751731111000796

6. Zhang L, Wang YX, Xiao X, Wang JS, Wang Q: Effects of zinc glycinate on productive and reproductive performance, zinc concentration and antioxidant status in broiler breeders. *Biol Trace Elem Res,*178, 320-326, 2017. DOI: 10.1007/s12011-016-0928-4

7. Chen F, Jiang Z, Jiang S, Li L, Lin X, Gou Z, Fan Q: Dietary vitamin A supplementation improved reproductive performance by regulating ovarian expression of hormone receptors, caspase-3 and Fas in broiler breeders. *Poult Sci,* 95, 30-40, 2016. DOI: 10.3382/ps/pev305

8. El-Husseiny OM, Abd-Elsamee MO, Omara II, Fouad AM: Effect of dietary zinc and niacin on laying hens performance and egg quality. *Int J Poultry Sci,* 7, 757-764, 2008. DOI: 10.3923/ijps.2008.757.764

9. Yair R, Uni Z: Content and uptake of minerals in the yolk of broiler embryos during incubation and effect of nutrient enrichment. *Poult Sci,* 90, 1523-1531, 2011. DOI: 10.3382/ps.2010-01283

10. Soni N, Mishra SK, Swain R, Das A, Chichilichi B, Sethy K: Bioavailability and immunity response in broiler breeders on organically complexed zinc supplementation. *Food Nutr Sci,* 4, 1293-1300, 2013. DOI: 10.4236/fns.2013.412166

11. Pearce J, Jackson N, Stevenson MH: The effects of dietary intake and of dietary concentration of copper sulphate on the laying domestic fowl: Effects on some aspects of lipid, carbohydrate and amino acid metabolism. *Br Poult Sci,* 24, 337-348, 1983. DOI: 10.1080/00071668308416748

12. Oviedo-Rondon EO, Leandro NM, Ali R, Koci M, Moraes V, Brake J: Broiler breeder feeding programs and trace minerals on maternal antibody transfer and broiler humoral immune response1. *J Appl Poult Res,* 22, 499-510, 2013. DOI: 10.3382/japr.2012-00708

13. Moraesa VMB, Oviedo-Rondón EO, Leandro NSM, Winelanda MJ, Malheirosa RD, Eusebio-Balcazara P: Broiler breeder trace mineral nutrition and feeding practices on embryo progeny development. *Avian Biol Res,* 4 (3): 2011 122-132, 2011.

14. Favero A, Vieira SL, Angel CR, Bess F, Cemin HS, Ward TL: Reproductive performance of Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. *J Appl Poult Res,* 22, 80-89, 2013. DOI: 10.3382/japr.2012-00607

15. Favero A, Vieira SL, Angel CR, Bos-mikich A, Lothhammer N, Taschetto D, Cruz RFA, Ward TL: Development of bone in chick embryos from Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. *Poult Sci,* 92, 402-411, 2013. DOI: 10.3382/ps.2012-02670

16. Abbaspour N, Hurrel R, Kelishadi R: Review on iron and its importance for human healt: *J Res Med Sci,* 19 (2:) 164-174, 2014.

17. Renema RA: Reproductive responses to Sel-Plex® organic selenium in male and female broiler breeders: Impact on production traits and hatchability. *Poult Indus,* https://en.engormix.com/poultry-industry/ articles/ sel-plex-organic-selenium-in-male-female-broiler-breeders-t33862.htm; *Accessed:* 21.06.2019.

18. Zia MW, Khalique A, Naveed S, Hussain J: Organic and inorganic selenium in poultry: A review. *Indian J Anim Res,* 52 (4): 483-489, 2018. DOI: 10.18805/ijar.v0iOF.6837

19. Ross: Parentstock Performance Objectives, Ross 308 FF-2016. www.aviagen.com; *Accessed:* 24.06.2016.

20. McQuoid, D: Operation of multiple-stage and single-stage incubation systems. *World Poult,* 18 (2): 18-22, 2000.

21. Ross: Broiler 308 Nutrition, Specifications, 2014; www.aviagen.com; *Accessed:* 24.05.2014.

22. Penuela A, Aureliano P: Characterization of embryonic mortality in broilers. *Rev MVZ Córdoba.* 23 (1): 6500-6513, 2018. DOI: 10.21897/rmvz.1245

23. Sahin EH, Sengor E, Yardimci M, Cetingul S: Relationship between pre-incubation egg parameters from old breeder hens, egg hatchability and chick weight. *J Anim Vet Adv,* 8 (1): 115-119. 2009.

24. SAS Institue: SAS User's Guide. Statistics. Version. 5th edn., SAS Institue Inc., Cary, NC.USA, 2005.

25. Świątkiewicz S, Arczewska-Włosek A, Józefiak D: The efficacy of organic minerals in poultry nutrition: review and implications of recent studies. *Worlds Poult Sci J,* 70, 475-485, 2014. DOI: 10.1017/S0043933914000531

26. Aksu DS, Aksuy T, Onel SE: Does inclusion at low levels of organically complexed minerals versus inorganic forms create a weakness in performance or antioxidant defense system in broiler diets? *Int J Poult Sci,* 11 (10): 666-672, 2012. DOI: 10.3923/ijps.2012.666.672

27. Swinkels JWGM, Kornegay ET, Verstegen MWA: Bology of zinc and biological value of dietary organic zinc complexes and chelates. *Nutr Res Rev,* 7, 129-149, 1994. DOI: 10.1079/NRR19940009

28. Nys Y, Hincke MT, Arisa JL, Carscia-Ruiz JM, Solomon SE: Avian egg shell mineralization. *Poult Avi Biol Rev.* 10,143-166, 1999.

29. Georgievski VI: Mineral Nutrition of Animals. 475, Butterworts, London, Boston, 1982.

30. Chowdhury SD: Shell membrane system in relation to lathyrogen toxicity and copper deficiency. *Worlds Poult Sci,* 46, 153-169, 1990.

31. Taschetto D, Vieira SL, Angel CR, Stefanello C, Kindlein L, Ebbing MA, Simões ST: Iron requirements of broiler breeder hens. *Poult Sci*, 96, 3920-3927, 2017. DOI: 10.3382/ps/pex208

32. Sahin T, Tasdemir AN: The effects of organic and inorganic zinc supplemented in breeder hens' rations on hatching traits and chick quality (II). *Vet Hekim Der Derg,* 88 (1): 3-12, 2017.

33. Zamani A, Rahmani HR, Pourreza J: Effect of different levels of manganese and zinc on performance traits and breaking eggs in laying hens. *Pakistan J Biol Sci,* 8 (7): 1035-1040, 2005. DOI: 10.3923/pjbs.2005.1035.1040

34. Chen S, Li T, Yeh R, Lien T, Huang C, Chen K: Impact of high dietary copper on the reproductive characteristics of laying hens and growth of newly hatched chicks. *J Chin Soc Anim Sci,* 43 (3): 11-24, 2014.

35. Attia YA, Abdalah AA, Zeweil HS, Bovera F, Tag El-Din AA, Araft MA: Effect of inorganic or organic copper additions on reproductive performance, lipid metabolism and morphology of organs of dual-purpose breeding hens. *Arch Geflügelk,* 75 (3): 169-178, 2011.

36. Yenice E, Mızrak C, Gültekin M, Atik Z, Tunca M: Effects of dietary organic or inorganic manganese, zinc, copper and chrome supplementation on the performance, egg quality and hatching characteristics of laying breeder hens. *Ankara Üniv Vet Fak Derg.* 62, 63-68, 2015. DOI: 10.1501/Vetfak_0000002659

37. Du Z, Hemken RW, Jackson JA, Trammell DS: Utilization of copper

in copper proteinate, copper lysine, and cupric sulfate using the rat as an experimental model. *J Anim Sci,* 74, 1657-1663, 1996.

38. Mondal MK, Das TK, Biswas P, Samanta CC, Bairagy B: Influence of dietary inorganic and organic copper salt and level of soybean oil on plasma lipids, metabolites and mineral balance of broiler chickens. *Anim Feed Sci Technol,* 139, 212-233, 2007. DOI: 10.1016/j.anifeedsci.2007.01.014

39. Lu L, Ji C, Luo XG, Liu B, Yu SX: The effect of supplemental manganese in broilers diets on abdominal fat deposition and meat quality. *Anim Feed Sci Technol,* 129, 49-59, 2006. DOI: 10.1016/j.anifeedsci.2005.12.005

40. Baloch Z, Yasmeen N, Pasha TN, Ahmad A, Taj MK, Khosa AN, Marghazani IB, Bangulzai N, Ahmad I, Hua YS: Effect of replacing inorganic with organic trace minerals on growth performance, carcass characteristics and chemical composition of broiler thigh meat. *Afr J Agric Res,* 12 (18): 1570-1575, 2017. DOI: 10.5897/AJAR2016.12104

41. Smith MO, Sherman LL, Miller LC, Robbins KR, Halley JT: Relative biological availability of manganese sulfate, and manganese monoxide in broilers reared at elevated temperatures. *Poult Sci,* 74, 702-707, 1995. DOI: 10.3382/ps.0740702

42. Bao YM, Choct M, Lji PA, Bruerton K: Optimal dietary inclusion of organically complexed zinc for broiler chickens. *Br Poult Sci,* 50, 95-102, 2009. DOI: 10.1080/00071660802590377

43. Hudson BP, Fairchild BD, Wilson JL, Dozier WA, Buhr RJ: Breeder age and zinc source in broiler breeder hen diets on progeny characteristics at hatching. *J Appl Poult Res,* 13, 55-64, 2004. DOI: 10.1093/japr/13.1.55

44. Rebel JMJ, Dam JTP, Zekarias B, Balk FRM, Post J, Flores A, Ter Huurne AAHM: Vitamin and trace mineral content in feed of breeders and their progeny: Effects of growth, feed conversion and severity of malabsorption syndrome of broilers. *Br Poult Sci,* 45 (2): 201-209, 2010. DOI: 10.1080/00071660410001715803

45. Sun Q, Guo Y, Ma S, Yuan J, An S, Li J: Dietary mineral sources altered lipid and antioxidant profiles in broiler breeders and posthatch growth of their offsprings. *Biol Trace Elem Res,* 145, 318-324, 2012. DOI: 10.1007/s12011-011-9196-5

46. Aksu T, Özsoy B, Aksu DS, Yörük MA, Gül M: The effects of lower levels of organically complexed zinc, copper and manganese in broiler diets on performance, mineral concentration of tibia and mineral excretion. *Kafkas Univ Vet Fak Derg,* 17 (1): 141-146, 2011. DOI: 10.9775/kvfd.2010.2735

Kafkas Univ Vet Fak Derg
26 (2): 181-186, 2020
DOI: 10.9775/kvfd.2019.22678

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Detection and Molecular Characterization of BCoVs Circulating in Central China Based on the Full-length Spike Gene

Zhihai SHI [1,3,#,a] Wenjia WANG [2,#,b] Zhaoxue XU [1,3] Mengmeng ZHAO [4,c] Yali LAN [1,3]

[#] *These authors contributed equally in the study*

[1] Institute of Animal Husbandry and Veterinary, Henan Academy of Agricultural Sciences, Zhengzhou, 450002, Henan, CHINA

[2] College of Pharmaceutical Engineering, Henan University of Animal Husbandry and Economy, Zhengzhou, 450046, Henan, CHINA

[3] Henan Key Laboratory of Farm Animal Breeding and Nutritional Regulation, Zhengzhou, 450002, Henan, CHINA

[4] School of Life Science and Engineering, Foshan University, Foshan, 528225, Guangdong, CHINA

[a] ORCID: 0000-0002-4083-5331; [b] ORCID: 0000-0002-3023-4698; [c] ORCID: 0000-0002-6589-4100

Article ID: KVFD-2019-22678 Received: 20.05.2019 Accepted: 13.11.2019 Published Online: 14.11.2019

How to Cite This Article

Shi Z, Wang W, Xu Z, Zhao M, Lan Y: Detection and molecular characterization of BCoVs circulating in central China based on the full-length spike gene. *Kafkas Univ Vet Fak Derg,* 26 (2): 181-186, 2020. DOI: 10.9775/kvfd.2019.22678

Abstract

Bovine coronavirus (BCoV) is prevalent throughout the world and is an important aetiological agent of diarrhoea in new-born calves. However, little is known about the genetic diversity and molecular epidemiology of BCoV in China. In this study, a total of 127 faecal samples from diarrhoeic dairy calves from nine cities in Henan Province, Central China, were collected between 2017 and 2018 and evaluated by RT-PCR for the N gene. BCoV was detected in 15% (19/127) of calves. In addition, the full-length sequence of the S gene from 13 representative BCoV strains was obtained and analysed. Sequencing results for the full-length S gene showed 97.3-97.5% nucleotide (nt) identity and 96.3-96.8% amino acid (aa) identity with the classical Mebus strain. Phylogenetic analysis based on the full-length S gene showed that the BCoV strains in this study clustered with Vietnamese and Cuban BCoV strains on a large branch of the tree. These results enrich the molecular characterization of the Chinese BCoV strains and provide the first large-scale epidemiological examination of the prevalence of BCoV in diarrhoeic calves in Henan Province, Central China.

Keywords: *BCoV, Spike gene, Phylogenetic analysis, China*

Orta Çin'de Sığır Koronavirusun Tam Uzunlukta Spike Genine Dayalı Tespiti ve Moleküler Karakterizasyonu

Öz

Sığır koronavirüs (BCoV) tüm dünyada yaygındır ve yeni doğmuş buzağılarda ishal olgularının etiyolojisinde önemli rol oynar. Bununla birlikte, Çin'de BCoV'nin genetik çeşitliliği ve moleküler epidemiyolojisi hakkında çok az şey bilinmektedir. Bu çalışmada, 2017 ile 2018 yılları arasında Orta Çin'in Henan eyaletindeki dokuz ilden ishalli süt buzağılarından toplam 127 adet dışkı örneği toplandı ve N geni için RT-PCR ile değerlendirildi. Buzağıların %15'inde (19/127) BCoV tespit edildi. Ek olarak, 13 temsilci BCoV suşundan S geninin tam uzunluktaki dizisi elde edildi ve analiz edildi. Tam uzunluktaki S geni için sıralama sonuçları, klasik Mebus suşu ile %97.3-97.5 nükleotit (nt) özdeşliği ve %96.3-96.8 amino asit (aa) özdeşliği gösterdi. Tam uzunluktaki S genine dayanan filogenetik analiz, bu çalışmada BCoV suşlarının, ağacın büyük bir dalında Vietnam ve Küba BCoV suşları ile kümelendiğini gösterdi. Bu sonuçlar, Orta Çin'in Henan Eyaletindeki ishalli buzağılarda BCoV suşlarının moleküler karakterizasyonunu zenginleştirerek BCoV prevalansının ilk büyük ölçekli epidemiyolojik incelenmesini sağlamış oldu.

Anahtar sözcükler: *BCoV, Spike geni, Filogenetik analiz, Çin*

INTRODUCTION

Bovine coronavirus (BCoV) is a lineage A member of the species *Betacoronavirus*, belonging to the family *Coronaviridae* in the order *Nidovirales*, as assigned by the International Committee on Taxonomy of Viruses (ICTV) [1]. *Betacoronaviruses* have a broad range of hosts, such as humans (Middle East respiratory syndrome coronavirus,

İletişim (Correspondence)
+86-371-65714519
mzhao8@tulane.edu (M. Zhao), yali2005haonan@sina.com (Y. Lan)

MERS-CoV [2], and human coronavirus OC43, HCoV-OC43), mice (mouse hepatitis coronavirus, MHV), and horses (equine coronavirus [3], ECoV). BCoV infection can cause mild to severe diarrhoea in new-born calves, winter dysentery in adult cattle and respiratory disorders in cattle of all ages [4-6], which causes dramatic a reduction in milk production in dairy herds and loss of body condition in both calves and adult cattle, accompanied by depression and anorexia, leading to serious economic losses [7]. BCoV has been identified in many countries worldwide.

Bovine coronavirus has a single-stranded, positive-sense RNA genome that is 32 kb in length, and is the largest virus among known RNA viruses. BCoV is an enveloped and pleomorphic virus with a diameter ranging from 100 to 120 nm, consisting of five major structural proteins designated the nucleocapsid (N) protein, membrane (M) protein, small envelope (E) protein, haemagglutinin-esterase (HE) protein and spike (S) protein [8]. The S glycoprotein of BCoV forms a club-shaped structure on the viral surface and has an important role in the process of virus invasion and fusion. It cleaves two subunits, termed S1 and S2, at amino acid position 768 [9]. During virus entry, S1 is responsible for binding sugar on the host cell surface for viral attachment and inducting of neutralizing antibody expression and haemagglutinin activity in the host species [9]. S2 is a transmembrane protein that fuses the host and viral membranes, allowing viral genomes to enter host cells. The variation in host range and tissue tropism of coronaviruses is largely related to variations in the S protein [10].

Bovine coronavirus was first reported in the USA in 1972 from enteritic neonatal calves and termed the Mebus strain. Subsequently, the presence of BCoV has extended to other areas of the world. In the 1980s, BCoV infections occurred in the cattle population in China [11]. However, limited information is available about BCoV circulating in Henan Province, Central China. Therefore, we sought to identify the frequency of BCoV infection among calves in Henan Province and investigate the genetic evolution of Henan BCoVs.

MATERIAL and METHODS

Between 2017 and 2018, diarrhoeic faecal samples (n=127) were collected from calves at 14 different farms from nine cities in Henan Province [An Yang (AY), He Bi (HB), Qin Yang (QY), Yuan Yang (YY), Lan Kao (LK), Song Xian (SX), Lu Shi (LS), Shang Cai (SC) and Bi Yang (BY)]. All calves were less than 4 months old. BCoV-positive faeces and the full-length S gene were diagnosed and amplified using gene-specific primers *(Table 1)*. The resulting sequence data were edited using Lasergene 7.0 Alignment Editor, and the sequence identities of the nucleotide and deduced amino acid sequences of the field strains in this study were performed using Clustal W software. A phylogenetic tree was constructed based on the deduced amino acid sequences encoded by the full-length S gene using the neighbour-joining (NJ) method with 1.000 bootstrap replicates in MEGA software, version 7.0. The NJ tree was visualized using Figtree 1.4.3 [12]. The positively selected sites were evaluated using maximum-likelihood (ML) method implemented in MEGA. A site model was selected for the positively selected sites, and the parameters under seven different codon substitution models and their performances were evaluated using likelihood ratio tests (LRTs) implemented in EasyCodeML 1.2 [13]. The animal experiments were carried out according to the Animal Experiment Committee of Henan Academy of Agricultural Sciences (Approval number SYXK 2014-0007). All animals

Table 1. *Primer pairs used to amplify the partial N and full-length S genes*

Primer Name	Sequence (5′→3′)	Size (bp)	Genome Position	Reference
NF	CCGATCAGTCCGACCAATC	460	N gene 80-539	[14]
NR	TAGTCGGAATAGCCTCATCGC			
S1F	ATGTTTTTGATACTTTTAATTTCC	920	S gene 1-920	[15]
S1R	ACACCAGTAGATGGTGCTAT			
S2F	GGGTTACACCTCTCACTTCT	769	S gene 782-1550	[15]
S2R	GCAGGACAAGTGCCTATACC			
S3F	CTGTCCGTGTAAATTGGATG	828	S gene 1459-2286	[15]
S3R	TGTAGAGTAATCCACACGT			
S4F	TTCACGACAGCTGCAACCTA	872	S gene 2151-3022	[15]
S4R	CCATGGTAACACCAATCCCA			
S5F	CCCTGTATTAGGTTGTTTAG	916	S gene 2691-3606	[15]
S5R	ACCACTACCAGTGAACATCC			
S6F	GTGCAGAATGCTCCATATGGT	653	S gene 3439-4092	[15]
S6R	TTAGTCGTCATGTGATGTTT			

received humane care in compliance with good animal practices according to the animal ethics procedures and guidelines of China.

RESULTS

Of the 127 faecal samples from the calves with diarrhoea, 19 (14.96%) were positive for BCoV. In terms of the geographical distribution, the percentages of BCoV samples by state were as follows: LK [4.72% (6/127)]; YY [3.94% (5/127)]; SC [4.72% (6/127)]; BY [0.79% (1/127)]; and LS [0.79% (1/127)] *(Fig. 1)*. These results indicate that BCoV is widely disseminated in cattle herds in Henan Province.

The full-length S gene was successfully obtained in 13 of the 19 positive samples, and they were designated SC-1, SC-2, SC-3, SC-4, SC-5, SC-6, LK-1, LK-2, YY-1, YY-2, YY-3, YY-4 and LS-1. Sequence comparisons of all 13 S genes in this study revealed that they share 99.5-100% nt identity and 98.9-100% aa identity with each other and 97.3-97.5% nt identity and 96.3-96.8% aa identity with the Mebus strain. They also share 98.6-98.9% nt identity and 98.4-99.3% aa identity with the other 7 Vietnamese BCoV S genes reported previously and 98.6% nt identity and 98.3% aa identity with the other two Chinese strains, AKS-01 from Xinjiang Province and HLJ-14 from Heilongjiang Province, respectively.

A phylogenetic tree based on the full-length S gene sequences using the NJ method showed that all 13 S genes from this study clustered on an independent small branch with each other, and clustered with 7 Vietnamese and 4 Cuban BCoV strains on a large branch of the tree *(Fig. 2)*.

The analysis of the predicted S proteins revealed that all 13 S genes from this study had 4092 nucleotides, encoded 1363 aa residues, and had a molecular weight of approximately 150 kDa. The S1 and S2 subunits were formed at the cleavage site aa 768, and their molecular weights were approximately 86 and 65 kDa, respectively. A total of 83 and 47 polymorphic nucleotides corresponding to 40 and 22 aa changes at 43 and 24 distinct sites were identified in the S1 and S2 subunits of the strains identified in this study compared with the Mebus strain, respectively. Of these amino acid changes, 19 were unique, while 43 were shared with other reference strains reported previously *(Fig. 3)*. In addition, the S1 N-terminal domain (S1-NTD, aa 15-298), which was shown to function as a receptor-binding domain (RBD) in BCoV, had a total of 20 aa changes in the strains of this study. In addition, the S1A and the S1B immunoreactive domains identified within the amino acids 351-403 and the amino acids 517-621 had 1 and 4 aa changes, respectively, in the strains in this study compared with the Mebus strain. Additionally, compared with the sequences of other BCoV S genes, six sequences of S genes from Shangcai city BCoV isolates in this study had unique N509H, T352I, I640I, S977A, and C1331F aa changes *(Fig. 3)*. No frameshifts, deletions or insertions were observed in the S gene sequences of strains in this study.

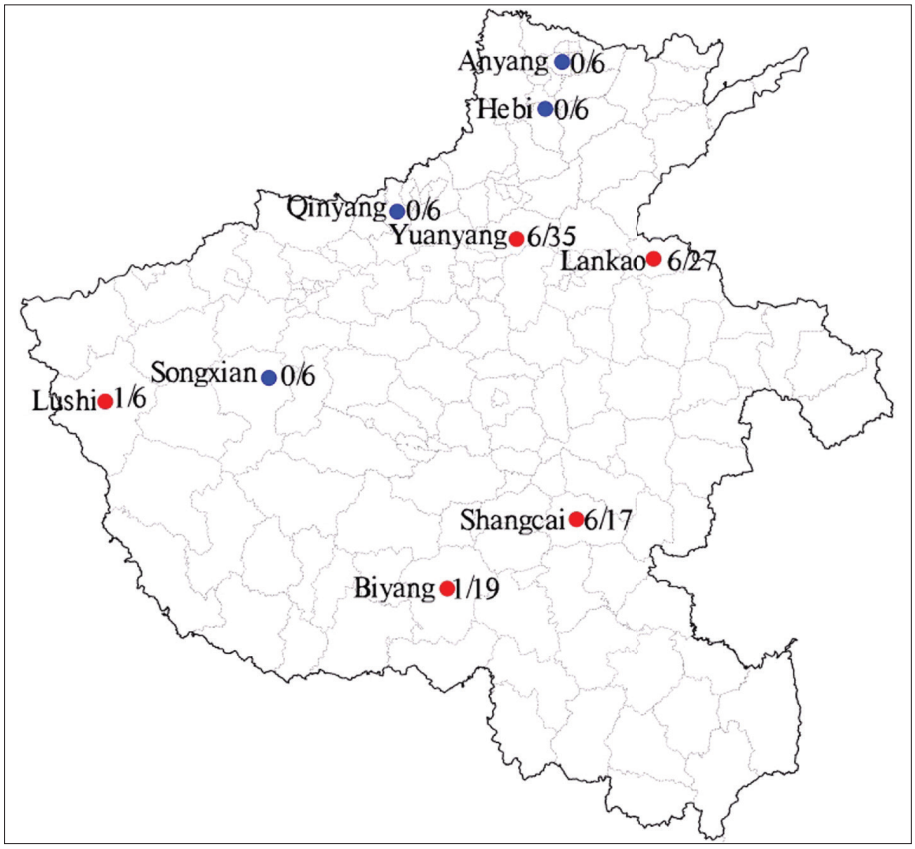


Fig 1. Map of Henan showing the number of samples and the geographic distribution of the sample collection sites. The red solid dot indicates an area where positive samples were located, and the blue solid dot indicates an area where no positive samples were located

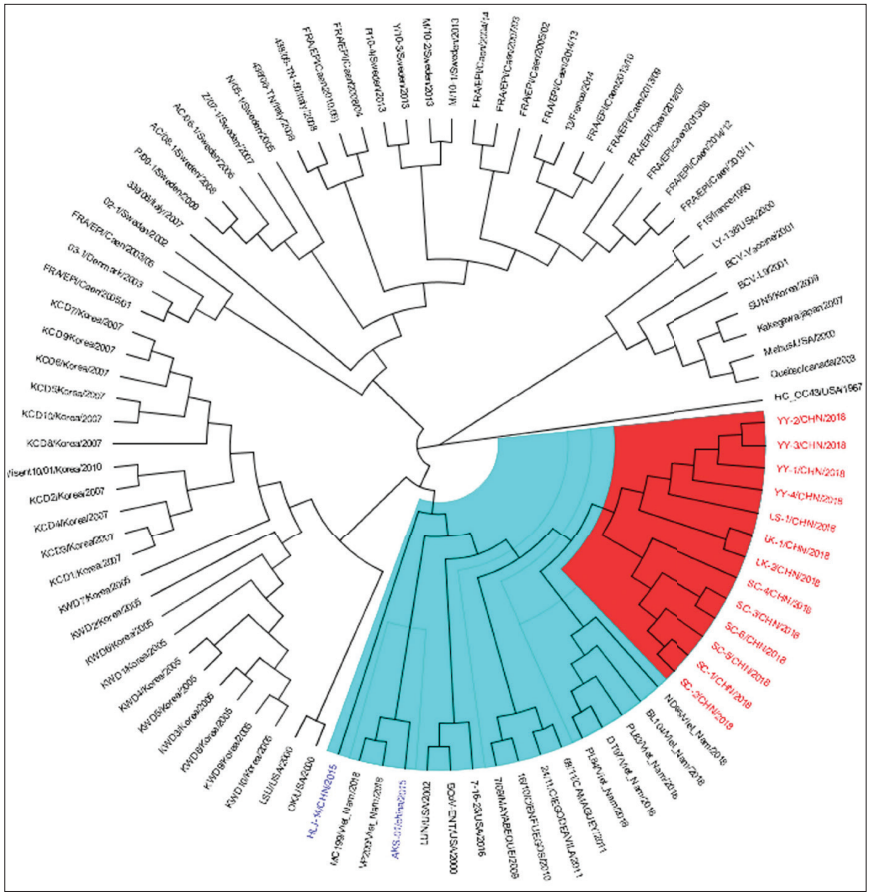


Fig 2. Phylogenetic tree generated by neighbour-joining analysis of the genetic distance in the deduced amino acid sequences of the full-length S gene of BCoV strains. The strains in this study are marked in red, and other Chinese BCoV strains are marked in blue

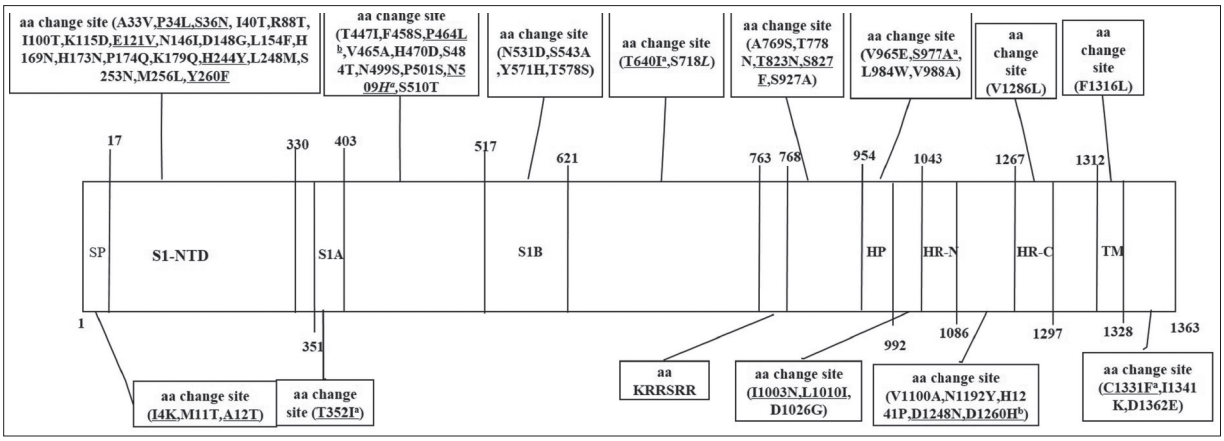


Fig 3. Amino acid variants of the 13 full-length S genes in this study. The unique aa changes in this study are underlined; [a] aa changes in six strains from Shangcai city; [b] aa changes in two strains from Lankao city

Likelihood ratio test analysis at the posterior probability p>95% level identified 11 positively selected sites. Of these 8 are in S1 and 3 are in S2. The following sites were under positive selection: 11, 115, 179, 499, 501, 524, 539, 716, 1235, 1350, and 1360 *(Table 2)*.

DISCUSSION

Bovine coronavirus, an important causative agent of diarrhoea in calves, is responsible for severe economic losses in the global farming industry. One recent study reported that BCoV infections are endemic in China and that the prevalence of BCoV in Henan Province is 46.7% [16], but the percentage in our study was 14.96%, which is lower than that in the previous study. The reasons for the difference in the detection rates of BCoV in calves in the same province could be explained by differences in sample number and sampling area. Fifteen samples from 3 farms were collected for detection in that study, while in our study, we screened 127 diarrhoeic faecal samples from calves, 19 of which were positive for BCoV. These positive samples were distributed across 10 of 14 farms across five different cities in the major

Table 2. *Parameter estimates and log-likelihood values under models of variable o-ratios among sites*

Model	Parameter Estimates	Likelihood Scores	Positively Selected Sites*
M0: one-ratio	ω=0.111175	-13568.203652	Not allowed
M1a: nearly neutral	ω0=0.04, ω1=1, (p0=0.91, p1=0.092)	-13336.145397	Not allowed
M2a: positive selection	ω0=0.04, ω1=1, ω2=1, (p0=0.91, p1=0.055, p2=0.038)	-13336.000953	None
M3: discrete	ω0=0.02, ω1=0.45, ω2=1.88, (p0=0.844, p1=0.13, p2=0.026)	-13325.821205	None
M7: β	p=0.041, q=0.217	-13385.024380	Not allowed
M8: $\beta+\omega_s>1$	p0=0.96, p1=0.042, p=0.047, q=0.335, ω=1.504	-13328.064083	11, 115, **179,** 499, 501, 524, 539, 716, 1235, 1350, 1360
M8a: $\beta+\omega_s=1$	p0=0.931, p1=0.069, p=0.302, q=5.403, ω=1	-13331.696625	Not allowed

** Sites with a posterior probability >95% having ω>1 and p>99% are in boldface*

dairy cattle production areas of Henan Province. This study is the first large-scale epidemiological examination to determine the prevalence of BCoV in diarrhoeic calves in Henan Province. The results showed that BCoV is widely disseminated in cattle herds in Henan Province.

The 13 Henan BCoV strains sequenced in this study show a higher similarity with the other seven Vietnamese BCoV strains, causing them to cluster as a unique clade in the phylogenetic tree *(Fig. 2)*. We also identified a high similarity between our strains and 2 other Chinese BCoV strains in distant regions of China, such as Heilongjiang and Xinjiang Provinces, implying that certain strains may have the potential to spread directly or indirectly to distant regions. These results also suggest that these BCoV strains were part of the main transmission chains in dairy herds in Asia. Interestingly, we found six strains from Shangcai city clustered in a smaller branch in the phylogenetic tree with strains from Lankao and Yuanyang cities *(Fig. 2)*. Compared with the BCoV S genes of other strains, the S genes of strains from these different cities have their own unique aa substitution changes, such as T352I, N509H, I640I, S977A, and C1331F aa changes in the Shangcai strains and P464L and D1260H aa changes in the Lankao strains *(Fig. 3)*. These results indicated that viral lineages can form natural groups based on geographical location [17]. These results may be linked to factors such as geographical distribution, different breeds and breeding systems and animal marketing [18].

Six BCoV strains from Shangcai city had the unique aa substitution S977A in the fusion peptide (FP) of the S2 subunit, and four BCoV strains from Yuanyang city in this study were observed to have the substitution V1285L located in the heptad repeat region C (HR-C). FP and HR-C are crucial for the fusion on viral and host cell membranes [19]. Membrane fusion is mediated by a major conformational rearrangement that exposes the fusion peptide and results in the formation of a six-helix bundle (6HB) [20], bringing the viral and host membranes together to fuse. The core of the 6HB is a triple-stranded coiled coil, and the HR-C elements pack within the grooves of the coiled coil in an antiparallel direction. Thus, aa substitutions in these regions may affect the interaction between the coiled-coil structure and the host cell receptor. Moreover, several reports have suggested that variation in the S2 subunit [21], particularly in FP and HR-C, determine host range expansion. Therefore, more sequence data and experimental studies are required to clarify the important role of these amino acid changes in the S2 subunit of BCoV.

The S protein is a major neutralizing antigen of BCoV. Researchers have previously confirmed that a single aa change within the S1B domain of the S protein of BCoV can confer resistance to virus neutralization [9]. In this study, the polymorphism T352I was identified in the S1A domain in only six strains from Shangcai city *(Fig. 3)*. However, whether this amino acid change affects the antigenicity of the virus is not known. Thus, the role of the T352I mutation in immunological properties requires further study.

In this study, two and five strong positive selection sites were detected within the receptor-binding domain (RBD) and an epitopic fragment of the S1 subunit of BCoV, spanning aa residues 15-298 and 324-720, respectively. Viral RBDs can recognize different receptors through structural variations and are a critical determinant of viral host ranges [19]. The epitopic fragments have been previously recognized using monoclonal antibodies (mAbs) [22]. Taken together, the strong positively selected sites within the S protein may be associated with the immune response and receptor binding and would thus be important in future BCoV vaccine development.

In conclusion, the results of our study have shown that BCoVs are circulating widely in dairy calves in Henan Province, Central China, and that most of these strains have unique evolutionary patterns based on our sequence analysis of the full-length S genes. Our findings will enrich the current understanding of the molecular characterization of BCoVs in China.

Funding

This work was funded by the Key Program for Science and Technology Development of Henan (192102110074), the National Key Research and Development Program of China

(2018YFD0501700), the National beef cattle industrial technology system (CARS-37), the Open Program for Buffalo research of Guangxi (SNKF-2015-03), as well as the National Natural Science Foundation of China (NSFC) (grants 31902279).

REFERENCES

1. Castells M, Giannitti F, Caffarena RD, Casaux ML, Schild C, Castells D, Riet-Correa F, Victoria M, Parreno V, Colina R: Bovine coronavirus in Uruguay: Genetic diversity, risk factors and transboundary introductions from neighboring countries. *Arch Virol,* 164 (11): 2715-2724, 2019. DOI: 10.1007/s00705-019-04384-w

2. Aleanizy FS, Mohmed N, Alqahtani FY, Mohamed RAEH: Outbreak of Middle East respiratory syndrome coronavirus in Saudi Arabia: A retrospective study. *BMC Infect Dis,* 17:23, 2017. DOI: 10.1186/s12879-016-2137-3

3. Giannitti F, Diab S, Mete A, Stanton JB, Fielding L, Crossley B, Sverlow K, Fish S, Mapes S, Scott L, Pusterla N: Necrotizing enteritis and hyperammonemic encephalopathy associated with equine coronavirus infection in equids. *Vet Pathol,* 52 (6): 1148-1156, 2015. DOI: 10.1177/0300985814568683

4. Singasa K, Songserm T, Lertwatcharasarakul P, Arunvipas P: Molecular and phylogenetic characterization of bovine coronavirus virus isolated from dairy cattle in Central Region, Thailand. *Trop Anim Health Prod,* 49 (7): 1523-1529, 2017. DOI: 10.1007/s11250-017-1358-9

5. Gunn L, Collins PJ, O'Connell MJ, O'Shea H: Phylogenetic investigation of enteric bovine coronavirus in Ireland reveals partitioning between European and global strains. *Irish Vet J,* 68:31, 2015. DOI: 10.1186/s13620-015-0060-3

6. Gomez DE, Arroyo LG, Poljak Z, Viel L, Weese JS: Detection of bovine coronavirus in healthy and diarrheic dairy calves. *J Vet Intern Med,* 31 (6): 1884-1891, 2017. DOI: 10.1111/jvim.14811

7. Ribeiro J, Lorenzetti E, Alfieri AF, Alfieri AA: Molecular detection of bovine coronavirus in a diarrhea outbreak in pasture-feeding Nellore steers in southern Brazil. *Trop Anim Health Prod,* 48 (3): 649-653, 2016. DOI: 10.1007/s11250-015-0975-4

8. Masters PS: The Molecular biology of coronaviruses. *Adv Virus Res,* 66 (1): 193-292, 2006. DOI: 10.1016/S0065-3527(06)66005-3

9. Yoo D, Deregt D: A single amino acid change within antigenic domain II of the spike protein of bovine coronavirus confers resistance to virus neutralization. *Clin Diagn Lab Immunol,* 8 (2): 297-302, 2001.

10. Gallagher TM, Buchmeier MJ: Coronavirus spike proteins in viral entry and pathogenesis. *Virology,* 279 (2): 371-374, 2001. DOI: 10.1006/viro.2000.0757

11. Lu CP, Yao HC, Eichhorn W: Coronavirus as an agent of neonatal calf diarrhea in a Chinese dairy cattle farm. *Zentralbl Veterinarmed B,* 38 (1-10): 473-476, 1991. DOI: 10.1111/j.1439-0450.1991.tb00898.x

12. Rambaut A: FigTree v1.4.3. 2009. http://tree.bio.ed.ac.uk/software/figtree/; *Accessed:* 25.03.2014.

13. Gao F, Chen C, Arab DA, Du Z, He Y, Ho SYW: EasyCodeML: A visual tool for analysis of selection using CodeML. *Ecol Evol,* 9 (7): 3891-3898, 2019. DOI: 10.1002/ece3.5015

14. Lojkić I, Krešić N, Šimić I, Bedeković T: Detection and molecular characterisation of bovine corona and toroviruses from Croatian cattle. *BMC Vet Res,*11:202, 2015. DOI: 10.1186/s12917-015-0511-9

15. Park SJ, Jeong C, Yoon SS, Choy HE, Saif LJ, Park SH, Kim YJ, Jeong JH, Park SI, Kim HH, Lee BJ, Cho HS, Kim SK, Kang MI, Cho KO: Detection and characterization of bovine coronaviruses in fecal specimens of adult cattle with diarrhea during the warmer seasons. *J Clin Microbiol,* 44 (9): 3178-3188, 2006. DOI: 10.1128/JCM.02667-05

16. Keha A, Xue L, Yan S, Yue H, Tang C: Prevalence of a novel bovine coronavirus strain with a recombinant hemagglutinin/esterase gene in dairy calves in China. *Transbound Emerg Dis,* 66 (5): 1971-1981, 2019. DOI: 10.1111/tbed.13228

17. Dennis AF, Mcdonald SM, Payne DC, Slavica MR, Esona MD, Edwards KM, Chappell JD, Patton JT: Molecular epidemiology of contemporary G2P[4] human rotaviruses cocirculating in a single U.S. community: Footprints of a globally transitioning genotype. *J Virol,* 88 (7): 3789-3801, 2014. DOI: 10.1128/JVI.03516-13

18. de Mira Fernandes A, Brandão PE, Dos Santos Lima M, de Souza Nunes Martins M, da Silva TG, da Silva Cardoso Pinto V, de Paula LT, Vicente MES, Okuda LH, Pituco EM: Genetic diversity of BCoV in Brazilian cattle herds. *Vet Med Sci*, 4, 183-189, 2018. DOI: 10.1002/vms3.102

19. Li F: Structure, Function, and evolution of coronavirus spike proteins. *Annu Rev Virol,* 3 (1): 237-261, 2016. DOI: 10.1146/annurev-virology-110615-042301

20. Forni D, Filippi G, Cagliani R, Gioia LD, Pozzoli U, Aldaghri N, Clerici M, Sironi M: The heptad repeat region is a major selection target in MERS-CoV and related coronaviruses. *Sci Rep,* 5:14480, 2015. DOI: 10.1038/srep14480

21. Graham RL, Baric RS: Recombination, reservoirs, and the modular spike: Mechanisms of coronavirus cross-species transmission. *J Virol,* 84 (7): 3134-3146, 2010. DOI: 10.1128/JVI.01394-09

22. Yoo DW, Parker MD, Song J, Cox GJ, Deregt D, Babiuk LA: Structural analysis of the conformational domains involved in neutralization of bovine coronavirus using deletion mutants of the spike glycoprotein S1 subunit expressed by recombinant baculoviruses. *Virology,* 183 (1): 91-98, 1991. DOI: 10.1016/0042-6822(91)90121-Q

Kafkas Univ Vet Fak Derg
26 (2): 187-192, 2020
DOI: 10.9775/kvfd.2019.22680

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Assessment of Regulations on Occupational Health and Safety in Agriculture in Turkey from the Point of Livestock Raising and Veterinary Medicine

Ayşe MENTEŞ GÜRLER [1,a] Şule SANAL [2,b] Gökhan ASLIM [3,c]

[1] Harran University, Faculty of Veterinary Medicine, Department of History of Veterinary Medicine and Deontology, TR-63200 Şanlıurfa - TURKEY
[2] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of History of Veterinary Medicine and Deontology, TR-55139 Samsun - TURKEY
[3] Selçuk University, Faculty of Veterinary Medicine, Department of History of Veterinary Medicine and Deontology, TR- 42003 Konya - TURKEY
[a] ORCID: 0000-0001-8402-7291; [b] ORCID: 0000-0002-5703-5752; [c] ORCID: 0000-0001-5976-8186

Article ID: KVFD-2019-22680 Received: 19.05.2019 Accepted: 18.09.2019 Published Online: 18.09.2019

How to Cite This Article

Menteş Gürler A, Sanal Ş, Aslım G: Assessment of regulations on occupational health and safety in agriculture in Turkey from the point of livestock raising and veterinary medicine. *Kafkas Univ Vet Fak Derg,* 26 (2): 187-192, 2020. DOI: 10.9775/kvfd.2019.22680

Abstract

Agriculture is the second largest source of employment following the service sector in the whole world, and it is among the most dangerous sectors concerning diseases and premature death. Majority of the families working in the agriculture sector also deal with livestock raising (cattle, sheep and goat farming) or they live in the areas with a high possibility of contact with animals. There are various problems since the occupational health and safety services do not cover the ones working on their own behalf and due to problems in the delivery of health services appropriate for the rural areas. Veterinary physicians, who play an important role in animal health, public health, and environment, have also important tasks in the implementations of occupational health and safety in livestock raising in agriculture sector. The material of this study was comprised of national and international regulations concerning occupational health and safety. The implementations of regulations of the International Labor Organization (ILO) were evaluated in terms of livestock raising and veterinary medicine. As the conclusion, in Turkey, it was determined that the regulations about occupational health and safety in agriculture did not involve the veterinary physicians concerning both safety practice and job safety; it can be suggested that it should be updated.

Keywords: *Agriculture, Livestock raising, Veterinary medicine, Occupational health and safety*

Türkiye'de Tarımda İş Sağlığı ve Güvenliği Düzenlemelerinin Hayvan Yetiştiriciliği ve Veteriner Hekimliği Açısından Değerlendirilmesi

Öz

Tarım tüm dünyada hizmet sektöründen sonra ikinci büyük istihdam kaynağını oluşturmakta ve aynı zamanda hastalık ve erken ölüm açısından en tehlikeli sektörler arasında yer almaktadır. Tarımla uğraşan ailelerin büyük çoğunluğu aynı zamanda küçük ve büyük baş hayvancılık yapmakta ya da hayvanlara temasın yüksek olduğu yerlerde yaşamaktadırlar. İş sağlığı ve güvenliği hizmetlerinin kendi nam ve hesabına çalışanları kapsamaması ve kırsal alanlara uygun sağlık hizmeti sunumu sağlanmasındaki güçlükler nedeniyle de çeşitli sorunlar yaşamaktadırlar. Hayvan sağlığı, halk sağlığı ve çevrenin korunmasında önemli bir rol oynayan veteriner hekimler, tarım sektöründe de hayvan yetiştiriciliği alanında iş sağlığı ve güvenliği uygulamalarında önemli hizmetler yürütmektedirler. Çalışmanın materyalini iş sağlığı ve güvenliği ile ilgili Ulusal ve Uluslararası mevzuat oluşturdu ve ilgili mevzuat ile ILO'nun tarım sektörüne ilişkin sözleşmeleri hayvan yetiştiriciliği ve veteriner hekimliği uygulamaları açısından değerlendirildi. Sonuç olarak, Türkiye'de tarımda iş sağlığı ve güvenliği ile ilgili mevzuatın, hem iş güvenliği uzmanlığı hem de iş güvenliği açısından veteriner hekimleri kapsamadığı tespit edilmiş olup güncellenmesi gerektiği iddia edilebilir.

Anahtar sözcükler: *Tarım, Hayvan yetiştiriciliği, Veteriner hekim, İş sağlığı ve güvenliği*

INTRODUCTION

Agriculture is defined as "*a sector, in which (workers) work in open and closed environments, in various geographies and climatic conditions, and which is conducted by means of various types of machine, animal, plant, and production*". It is the second largest source of employment after the service sector in the whole world and it is accepted among

İletişim (Correspondence)
+90 414 3183917
mentesgurler@gmail.com

the most dangerous sectors concerning diseases and premature death [1].

Livestock raising, which is included into agriculture, involves many activities such as giving help at birth, feeding, sterilization, making treatments, herding, managing, insemination, and butchery, including a vast variety of types such as horse, cattle, sheep, goat, and poultry [1].

Health and safety implementations in the agriculture sector needed to be handled different from other businesses since its living environment and work environment is the same, and since all family members live and work together [2]. Majority of the families working in the agriculture sector also deal with livestock raising (cattle, sheep and goat farming) or they work in the areas with a high possibility of contact with animals [3].

However, the workers of the agriculture sector, which is generally comprised of small and medium sized family enterprises, have various problems since the occupational health and safety services do not cover the ones working on their own behalf and due to problems in the delivery of health services appropriate for the rural areas [2].

One of the most important occupational elements of agriculture and livestock raising, veterinary physicians have important roles in protection of animal health, public health, and environment. Additionally, they have important tasks concerning the implementations of occupational health and safety in livestock raising field in agriculture sector. Among the professional tasks of veterinary physicians are ensuring the food safety, fighting against and preventing zoonosis diseases, providing preventive services, struggling against biological and agricultural terror, protecting the ecosystem and environmental health, controlling the vaccine preventable diseases, healing the chronic diseases that negatively influence the health quality, and conducting medical research studies to contribute to the public health [4].

Although there is an intense agriculture and livestock raising activity in Turkey, the number of the epidemiological studies conducted on the health of the workers in agriculture is limited, and the first scientific symposium employed in this issue was held in Şanlıurfa province in 6-7 April, 2012. The subject of the Occupational Health and Safety week of the year 2012 was decided by the Ministry of Labor and Social Security as '*agriculture*'; institutional steps were taken in the ministry like establishment of joint advisory committee of occupational health and safety. Moreover, evaluation of occupational risks in agriculture and developing guides particular to agriculture projects were started by the ministry [5].

The Occupational Health and Safety Law[1] no. 6331, which imposed the obligation for the employers to employ job safety specialist, occupational physician, and other health personnel among the employee, entered into force in 2012.

In this study, it is aimed that evaluating the Occupational Health and Safety Law and the regulations of the International Labor Organization (ILO) about the agriculture sector in terms of livestock raising and veterinary medicine implementations.

MATERIAL and METHODS

The material of the study is comprised of national and international regulations about occupational health and safety. The Occupational Health and Safety Law no. 6331, the regulations introduced in this context, and the documents of the ILO about agriculture sector were examined in terms of livestock raising and veterinary medicine. Additionally, the literature in this subject was reviewed and an evaluation was made in context of the data obtained from relevant books, articles, and scientific studies.

RESULTS

The first aggreement of ILO C129[2] (*Labor Inspection (Agriculture) Convention*), concerning agriculture was signed in 1969 and came into force in 1972. Moreover, there are the regulations numbered 155[3], 161[4] and 187[5] regulating the issues concerning occupational health and safety in general working life. Additionally, particular to agriculture sector, with the regulations numbered "C184 - *Safety and Health in Agriculture Convention*"[6] and "R192 - *Safety and Health in Agriculture Recommendation*"[7] the issues concerning occupational health and safety in agriculture were regulated. The regulations numbered C129 and C184 have not been ratified in Turkey yet.

In Turkey, in the Occupational Health and Safety Law[a] no. 6331, the occupational safety specialists are defined as the supervisors, the graduates of engineering or faculties providing architecture education and technical staff, who are inspecting the working life in the Ministry and relevant institutions and who are authorized by the Ministry to be employed in the occupational health and safety field with the occupational safety specialty certificate. The occupational groups defined as *"technical personnel"* are the technical teachers, the ones with the title physicist, chemist, or biologist and the graduates of occupational health and safety programs of vocational high schools of universities. The workplaces were separated into three groups as "Very hazardous", "Hazardous", "Less hazardous", and the specialists as A, B, and C class.

[1] Official Gazette dated 30.06.2012 and numbered 28339.

[2] C129- Labor Inspection (Agriculture) Convention, 1969.

[3] Agreement about Occupational Health and Safety and Working Conditions numbered 155.

[4] Agreement About Health Services numbered 161.

[5] Framework Agreement About Developing the Occupational Health and Safety numbered 187.

[6] C184 - Safety and Health in Agriculture Convention, 2001.

[7] R192 - Safety and Health in Agriculture Recommendation, 2001.

In the 9th article of the law no. 6331 titled as *"Determining the hazard classes"*, it was stated that the hazard classes of the working places would be determined according to the notification of the ministry, and accordingly the *"Notification of Working Place Hazard Classes Concerning Occupational Health and Safety"*[8] was enacted. According to the "Statistical classification of economic activities in the European Community" system *(Nomenclature statistique des Activités* économiques *dans la Communauté Européenne-NACE)*, which is in the appendix of this notification, the coded activities concerning the animal production, animal products, and veterinary medicine are given in *Table 1, 2, 3, 4, 5, 6,* and *7*. Almost all of the activities given in the tables are within the "hazardous" class, necessitating the employment of a *"(B) Class Occupational Safety Specialist"*.

By the Ministry of Labor and Social Security, it was determined that a meeting was held titled *"Cooperation Meeting for Occupational Health and Safety in Agriculture"*

[8] Official Gazette dated 26.12.2012 and numbered 28509.

Table 1. *Hazard class list of animal production activities*

NACE Rev. 2. Code	NACE Rev. 2. Description	Hazard Class
01.4	Animal production	
01.41	Raising of dairycattle	
01.41.31	Raising of dairycattle (cattle and buffaloes)	Hazardous
01.42	Raising of other cattle and buffaloes	
01.42.09	Raising of other cattle and buffaloes (except dairycattle)	Hazardous
01.43	Raising of horses and other equines	
01.43.01	Raising of horses and other equines (donkey, mule or hinny etc.)	Hazardous
01.44	Raising of camels and camelids	
01.44.01	Raising of camels and camelids	Hazardous
01.45	Raising of sheep and goats	
01.45.01	Raising of sheep and goats (including production of unprocessed milk, hair, mohair, fleece, wool, etc.)	Hazardous
01.46	Raising of swine/pigs	
01.46.01	Raising of swine/pigs	Hazardous
01.47	Raising of poultry	
01.47.01	Raising of poultry (chicken, turkey, duck, goose and guinea fowl etc.)	Hazardous
01.47.02	Operation of poultry hatcheries	Hazardous
01.47.03	Egg production from poultry	Hazardous
01.49	Raising of other animals	
01.49.01	Bee-keeping and production of honey and beeswax (including royal jelly)	Hazardous
01.49.02	Raising of silk worms, production of silk worm cocoons	Hazardous
01.49.03	Raising and breeding of domesticated live animals (except fish) (cats, dogs, birds, hamsters etc.)	Hazardous
01.49.05	Raising ostriches	Hazardous
01.49.90	Raising and breeding of semi-domesticated or other live animals (other birds (except poultry), insects, rabbits and other fur animals, snails, worm farms, reptile farms, animal embryos etc.)	Hazardous
01.5	Mixed farming	
01.50	Mixed farming	
01.50.06	Mixed farming (combined production of crops and animals without a specialised production of crops or animals)	Hazardous

Table 2. *Hazard class list of support activities for animal production*

NACE Rev.2. Code	NACE Rev. 2. Description	Hazard Class
001.62	Support activities for animal production	
01.62.01	Herd menagement, agistment services, coop cleaning, sheep shearing, milking, farm animal boarding, activities of farriers etc. activities on a fee or contract basis	Hazardous
01.62.02	Herd testing services, poultry sterilization, artificial insemination etc. (including operation of poultry hatcheries)	Hazardous

Table 3. *Hazard class list of hunting, trapping and related service activities*		
NACE Rev. 2. Code	**NACE Rev. 2. Description**	**Hazard Class**
01.70	Hunting, trapping and related service activities	
01.70.01	Hunting and trapping on a non commercial basis (for food, fur, skin, or for use in research etc.) (except fishing)	Hazardous
01.70.02	Hunting and trapping on a commercial basis (for food, fur, skin, or for use in research etc.) (except fishing)	Hazardous

Table 4. *Hazard class list of manufacture of food products*		
NACE Rev.2. Code	**NACE Rev. 2. Description**	**Hazard Class**
C	MANUFACTURING	
10	Manufacture of food products	
10.1	Processing and preserving of meat and production of meat products	
10.11	Processing and preserving of meat	
10.11.01	Slaughtering cattle, sheep, goats ect. and processing of meat (abattoirs) (production of fresh, chilled or frozen meat, in carcasses and cuts)	Hazardous
10.12	Processing and preserving of poultry meat	
10.12.01	Processing of poultry meat (fresh or frozen) (including edible offal)	Hazardous
10.12.02	Operation of slaughterhouses engaged in killing, dressing or packing poultry	Hazardous
10.12.03	Rendering of edible poultry fats	Hazardous
10.12.04	Production of feathers and down (including skins)	Hazardous
10.13	Production of meat and poultry meat products	
10.13.01	Manufacturing uncooked meatballs and similar products produced from meat and poultry meat	Hazardous
10.13.02	Manufacturing of salted, dried or smoked products as sausage, salami, fermented sausages, bacon, roasting meat, canned meat, pickled meat, ham etc. produced from meat and poultry meat (except meals) production of dried, salted or smoked meat	Hazardous
10.13.03	Production of meat and offal meal (produced from meat and poultry meat)	Hazardous
10.13.04	Production of edible animal offal and fats from cattle, sheep, goats etc.	Hazardous
10.2	Processing and preserving of fish, crustaceans and molluscs	
10.20	Processing and preserving of fish, crustaceans and molluscs	
10.20.03	Preparation and preservation of fish, crustaceans and molluscs (freezing, drying, cooking, smoking, salting, immersing in brine, canning etc.)	Hazardous
10.20.04	Production of fish, crustacean and mollusc products (fish fillets, roes, caviar, caviar substitutes etc.)	Hazardous
10.20.05	Production of fish meal, flour and pellets (for human consumption)	Hazardous
10.20.06	Activities of vessels and boats engaged only in processing and preserving of fish	Hazardous
10.20.07	Production of uncooked fish dishes (fermented fish, fish pulp, fish cake etc.)	Hazardous
10.20.08	Production of meals, flour and pellets from fish, crustaceans, molluscs or other aquatic animals (unfit for human consumption) and production of other inedible products	Hazardous

in 28 February 2012 *"for determining the current state of the occupational health and safety in agriculture, collecting together the country-wide efforts in this field, determining the requirements, and share of information and experience among institutions"*; it was also determined that *"The Pilot Project for Occupational Health and Safety in Agriculture"* was started in 2 March 2012, in Şanlıurfa and Adana [6].

According to the cooperation protocol signed between the Institute for Occupational Health and Safety of Turkey and Institution of Occupational Safety and Health (IOSH) of England in 2012, a pilot project was prepared in order to determine the risks in agriculture and food sectors and to prepare guides. As a result of this project, the following guides were prepared [6-8]:

The guide for determining the occupational health and safety risks in livestock sector (Still-continuing),

The occupational health and safety guide in red meat and poultry processing plants,

The occupational health and safety guide in fruit and vegetable businesses,

The guide for determining the occupational health and safety risks in greenhouse production,

The occupational health and safety guide in milk and milk products sector,

The guide for evaluating the occupational health and

Table 5. *Hazard class list of manufacture of dairy products*		
NACE Rev. 2. Code	**NACE Rev. 2. Description**	**Hazard Class**
10.5	**Manufacture of dairy products**	
10.51	**Operation of dairies and cheese making**	
10.51.01	Manufacture of fresh liquid milk (pasteurised, sterilised, homogenised and/or ultra heat treated) (except production of raw mil kor milk powder)	Hazardous
10.51.02	Manufacture of cheese and curd	Hazardous
10.51.03	Milk powder, casein, milk sugar (lactose) and whey manufacturing (including solid or powdered milk and cream)	Less Hazardous
10.51.04	Manufacture of milk-based soft drinks (kefir, orchid etc.)	Less Hazardous
10.51.05	Manufacture of other products made from milk (butter, yogurt, buttermilk, cream, sour cream, etc.) (Including cream) (excluding whipped cream in solid or powder form)	Hazardous
10.52	**Manufacture of ice cream**	
10.52.01	Manufacture of ice cream (plain, with vegetables, with fruits etc.)	Less Hazardous
10.52.02	Manufacture of other edible ice such as sorbet	Less Hazardous

Table 6. *Hazard class list of manufacture of prepared animal feeds*		
NACE Rev. 2. Code	**NACE Rev. 2. Description**	**Hazard Class**
10.9	Manufacture of prepared animal feeds	
10.91	Manufacture of prepared feeds for farm animals	
10.91.01	Manufacture of prepared feeds for farm animals	Hazardous
10.92	Manufacture of prepared pet foods	
10.92.01	Manufacture of prepared pet foods (including dogs, cats, birds, fish etc.)	Hazardous

Table 7. *Hazard class list of Veterinary activities*		
NACE Rev. 2. Code	**NACE Rev. 2. Description**	**Hazard Class**
75	Veterinary activities	
75.0	Veterinary activities	
75.00	Veterinary activities	
75.00.02	Activities of veterinary hospitals (includes animal ambulance activities for pet animals)	Hazardous
75.00.04	Veterinary activities (other activities, outside from animal hospitals)	Hazardous

safety risks in open agricultural areas.

In the occupational health and safety guide for red meat and poultry processing plants, it was observed that possible hazards, risks and probable control measurements in production activities were determined, and risky actions that can cause serious injuries and accidents were indicated [7].

In the occupational health and safety guide in milk and milk products sector, it was emphasized that *"…based on the NACE code, (it) was prepared to inform the employers, Occupational Health and Safety Professionals, and workers about possible hazards and risks that they can face in the "10.5 Dairy Products" sector and about the measurements to be taken"*. In the *"Biological Factors"* chapter of the guide, it was reported that among the leading communicable diseases from animals to milk, and from milk to human beings were *"Tuberculosis, Brucella, and Salmonella"*; it was also stated that these diseases can spread to the workers, and the reproduction of microorganisms is inevitable particularly in the tanks that are used for collecting the raw milk [8].

The *"Cooperation Protocol for Occupational Health and Safety in Agriculture"* was signed on 19 March 2013, by the Ministry of Labor, Ministry of Food, Agriculture and Livestock, Ankara University, Çukurova University, and Harran University [5]. The aim of the Protocol was defined as *"to cooperate with the aim of establishing the culture of occupational health and safety, guiding employers and employees by means of protection from existing risks, monitoring the developments and disseminating good practice examples to the whole agricultural sector, which has a high risk for occupational health and safety"*. Under the Protocol, *"Joint Advisory Board of Occupational Health and Safety in agriculture (JAB)"* was created and decided to meet twice a year.

DISCUSSION

The duties and authorities of veterinary physicians were determined in the 5th article of *"The Law on the Implementation of the Veterinary Profession, Establishment and Duties of the Union and Chambers of Turkish Veterinary Physicians"* no.6343. In addition to diagnosing and treatment of animal diseases, among these duties and authorities are the production, analysis, and control of all animal products, vaccines, and medicines to be used in the field of animal health, preventing the infectious animal diseases and movements that will hamper the breeding and development of livestock in the country, and using the scientific knowledge in the zootechnics field. Therefore, all the activities stated in the *Tables 1-7*, are among the duties and authorities of veterinary physicians and all of them are within the "hazardous" class, necessitating the certificate of *(B) Class Occupational Safety Specialist*. Moreover, veterinary medicine was included into *(Table 7)* the hazardous working places as a profession. However, veterinary physicians are not among the occupational groups allowed for the *Occupational Safety Specialty*. In this context, necessary regulations should immediately be made, and veterinary physicians should also be included into these occupational groups.

Particularly for the health and safety implementations of the agriculture sector and considering its unique characteristic [2] that is different from other professions, it can be suggested that the legal regulations in Turkey should be restructured in line with the regulations and agreements designated by the ILO in the agriculture sector.

The *"Arrival and Butchering of the Livestock"* and *"Occupational Diseases"* chapters of the *"The occupational health and safety guide in red meat and poultry processing plants"* are the parts, where the support of veterinary physicians is necessary [7]. Similarly, particularly in the *"Biological Factors"* chapter of the *"The occupational health and safety guide in milk and milk products sector"* [8], the requirement was clearly revealed that the veterinary physicians should be among the occupational health and safety specialists. In the "*The guide for determining the occupational health and safety risks in livestock sector"*, which is still under preparation, it is considered that taking the opinions of the veterinary medicine education institutions, implementation units, and professional associations will be beneficial for the sector.

The inclusion of livestock raising and related activity fields and veterinary profession into the *"Cooperation Protocol for Occupational Health and Safety in Agriculture"* and *"Joint Advisory Board of Occupational Health and Safety in Agriculture"* will make contributions for more efficient occupational health and safety efforts.

In a study conducted by Aslım and Yaşar [9], it was determined that veterinary physicians face many occupational risks such as accidents, chemical components, infectious diseases, compulsion, injuries, sprains during their professional practice and it was reported that occupational health and safety should be a predetermined requirement for veterinary medicine. However, in the law no. 6331, agricultural workers and cultivators are included into the occupational health and safety regulations, but veterinary physicians, who are directly exposed to occupational diseases, zoonosis, and work accidents are excluded. It can be stated that it is necessary and very important that the required legal arrangements should immediately be designated in order to increase the safety in the profession of veterinary medicine, which is one of the most important occupational groups in terms of agriculture and livestock raising.

As the conclusion, it can be suggested that the regulations concerning the occupational health and safety should be updated so as to include veterinary physicians both in *occupational safety specialists* and *occupational safety*. Additionally, the role of veterinary physicians is very important for the protection of both animals and people who care for them from disease and other risks in animal husbandry. The awareness levels of the veterinary physicians should be increased about protecting both themselves and the people they work with from possible risks, and about taking necessary measurements against injuries and diseases.

REFERENCES

1. ILO: ILO Safety and health in agriculture. ILO code of practice. International Labour Office - Geneva: ILO, 327, 2011.

2. Donham KJ, Thelin A: Agricultural Medicine; Occupational and Environmental Health for the Health Professionals. Iowa, USA: Blackwell Publishing, 2006.

3. Anonymous: *I. Tarım Sağlığı ve Güvenliği Sempozyumu: Çevresel ve Mesleki Riskler.* 6-7 Nisan, Şanlıurfa, 2012.

4. Cevizci S, Erginöz E: İnsan sağlığı ile veteriner hekimlik uygulamalarının ilişkisi: "Veteriner Halk Sağlığı". *İstanbul Üniv Vet Fak Derg*, 34 (2): 49-62, 2008.

5. Menteş Gürler A, Şimşek Z (Eds): Tarımda İş Sağlığı ve Güvenliği Çalıştayı. Renk Matbaası, İstanbul, 2016.

6. ÇSGB: Tarımda İş Sağlığı ve Güvenliği Rehberi. 34, T.C. Çalışma ve Sosyal Güvenlik Bakanlığı Genel Yayın No: 44, Ankara (Tarihsiz).

7. ÇSGB: Kırmızı Et ve Kanatlı Hayvan İşleme Tesislerinde İş Sağlığı ve Güvenliği Rehberi, 62, Ankara (Tarihsiz).

8. ÇSGB: Süt Ürünleri İmalatı Sektöründe İş Sağlığı ve Güvenliği Rehberi. 36, Ankara, (Tarihsiz).

9. Aslım G, Yaşar A: Gıda Tarım ve Hayvancılık Bakanlığında görev yapan veteriner hekimlerin iş sağlığı ve güvenliği üzerine bir değerlendirme. *V. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu,* 25-27 Mayıs, Bildiri Özetleri Kitabı, s.86, Bursa, 2016.

Kafkas Univ Vet Fak Derg
26 (2): 193-200, 2020
DOI: 10.9775/kvfd.2019.22718

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Effects of Propolis Extracts on Biogenic Amine Production by Food-Borne Pathogens

Aykut BURGUT [1,a]

[1] Department of Animal Science, Faculty of Agriculture, Cukurova University, TR-01330 Adana - TURKEY

Article ID: KVFD-2019-22718 Received: 24.05.2019 Accepted: 02.10.2019 Published Online: 02.10.2019

How to Cite This Article

Burgut A: Effects of propolis extracts on biogenic amine production by food-borne pathogens. *Kafkas Univ Vet Fak Derg,* 26 (2): 193-200, 2020. DOI: 10.9775/kvfd.2019.22718

Abstract

The impacts of water and ethanolic extracts of propolis on growth of Gram- positive and Gram-negative food-borne pathogens and their biogenic amine production were investigated. Ethanolic extracts of propolis had lower minimum inhibitory concentration (MIC) than that of water extract. Undiluted water extract of propolis (100%) was more effective on growth inhibition of *Staphylococcus aureus* (29.5 mm) and *Klebsiella pneumoniae* (26.5 mm) than antibiotics. The effect of propolis on biogenic amine production showed a discrepancy depending on bacterial strains, specific amine and extract type. Water or ethanolic extracts of propolis induced about 81-fold lower histamine accumulation by *Yersinia enterocolitica*. The study results suggested that both propolis extracts tested could be used as antimicrobial as they inhibit biogenic amines which were toxically important, although both propolis extracts exerted variability even among Gram- positive or negative bacteria. Moreover, stimulation of serotonin production by bacteria in the presence of propolis extracts emphasized important aspects of propolis for utilization in foods.

Keywords: *Propolis, Food-Borne Pathogen, Antimicrobials, Histamine, Serotonin*

Propolis Ekstrelerinin Gıda Kaynaklı Patojenlerin Biyojen Amin Üretimi Üzerindeki Etkileri

Öz

Bu çalışmada sulu ve etanolik propolis ekstrelerinin Gram pozitif ve negatif gıda kaynaklı patojen bakteri gelişimi ve biyojen amin üretimleri üzerindeki etkileri incelenmiştir. Propolisin etanolik ektreleri sulu ekstrelerine kıyasla daha düşük minimum inhibisyon konsantrasyonuna (MIK) sahip olmuştur. Seyreltik olmayan sulu propolis ekstresi (%100) *Staphylococcus aureus* (29.5 mm) ve *Klebsiella pneumoniae* (26.5 mm) gelişimini engellemede antibiyotiğe kıyasla daha etkili olmuştur. Propolisin biyojen amin üretimindeki etkisi bakteriyel üye, spesifik amin ve ekstre tipine göre farklılıklar göstermiştir. Sulu ve etanolik propolis ekstresi *Yersinia enterocolitica* tarafından histamin üretimini 81 kat düşürmüştür. Araştırma sonucunda test edilen her iki propolis ekstresinin Gram pozitif ve negatif bakteriler üzerindeki farklı etkilerine ragmen, toksikolojik olarak önemli biyojen aminleri engellemesi bakımından antimikrobiyal olarak kullanılabileceğini göstermiştir. Ayrıca, serotonin üretimini teşvik etmesi yönüyle propolis ekstresinin gıdalarda kullanımının bir avantaj sağlayacağı gözlenmiştir.

Anahtar sözcükler: *Propolis, Gıda kaynaklı patojen bakteriler, Antimikrobiyaller, Histamin, Serotonin*

INTRODUCTION

Consumers are concerned about chemical added foods, a fact that enhanced the demand for natural preservatives, because of their teratogenicity, carcinogenicity and residual effects [1]. Propolis has been reported to be non- toxic to humans, if it is not taken at high concentrations [2] and generally recognised as safe-GRAS [3]. These properties make them *attractive* for many food *applications* as a natural preservative [4].

Propolis, known also as bee glue, is a natural dark-coloured, resinous sticky constituent. It is collected by honey bees by mixing their own waxes with resins from plants, and used as a protective agent against their enemies [5]. Raw propolis is known to have 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris [3]. Propolis consists of various compounds, such as polyphenols (flavonoids, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones), sesquiterpene

İletişim (Correspondence)
+90 322 3386084 Fax: +90 322 3386439
burguta@cu.edu.tr

quinones, coumarins, lignans, steroids, amino acids, aromatic acids and inorganic compounds [6].

Propolis exerts several biological properties involving antibacterial, antioxidant, antiviral, antifungal, anti-inflammatory, antitumoral, immunomodulatory, local anaesthetic and antimutagenic [7]. These properties make it suitable for use in the treatment of wounds and burns, sore throat, and stomach ulcer as well as medical devices, health foods, beverages, cosmetics, improving the growth performance of livestock, food preservation, food packaging and textile materials for biomedical application [8-10]. The uses of different concentrations of propolis extract significantly inhibited microbial growth on cheese, beef patties and fruit [11-13]. The antibacterial activity of extract of propolis against food-borne pathogens such as *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* was reported by Nedji and Loucif-Ayad [14]. The antibacterial activity of propolis and its extract against Gram-positive and Gram-negative bacteria have been indicated in many studies. Antibacterial activity of propolis was shown mostly against Gram-positive strains but had been limited or not against Gram-negative strains [15]. The antimicrobial properties are associated with the existence of flavonoid and phenol compounds, although their mechanism of action is not clear [16]. The antimicrobial impact of propolis depends on its source, chemical composition, extract concentration and extraction method [17]. Mediterranean propolis is characteristic by the high concentration of terpenoids [7].

Biogenic amines (BAs) are known as nitrogenous compounds of low molecular weight and crucial at low concentrations for natural metabolic and physiological roles in animals, plants, and microorganisms [18]. However, the existence of high levels of BAs, especially histamine, putrescine, cadaverine and tyramine in foods could lead basically allergic reactions in humans, as a result, cause difficulty in breathing, itching, rash, vomiting, fever, hypertension, even severe toxicological symptoms, migraine, brain haemorrhage, heart failure, and abdominal cramps [19]. The most commonly found BAs in foods are histamine, putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, spermine and spermidine. The simultaneous formation of BAs including putrescine, cadaverine, spermidine, spermine and agmatine interferes intestinal histamine-metabolizing enzymes and increase histamine poisoning [20]. These BAs can also react with nitrites to create potentially carcinogenic nitrosamines. Therefore, the presence of BAs in foods requires a great deal of attention [21].

Many bacterial genera including some food borne pathogens such as *Salmonella, Klebsiella, Enterococcus, Clostridium*, and *Bacillus* have an ability to decarboxylate amino acids [22]. BAs formation in food has been controlled primarily by preventing microbial growth. Thus, the prevention of BAs formation has mainly focused on inhibiting the growth of BAs forming bacteria. The demand for the use of natural products with high health benefits such as propolis as a food component is increasing [23]. Although many researches have investigated antibacterial activity of propolis, to the best *of our knowledge*, *there is no study* regarding their potential role on biogenic amine formation by bacteria. Thus, the aim of the study was to investigate the impact of two different extracts of propolis on growth of common food-borne pathogens and their biogenic amine production.

MATERIAL and METHODS

Food-borne Pathogens

Enterococcus faecalis ATCC29212, *Staphylococcus aureus* ATCC29213, *Klebsiella pneumoniae* ATCC700603, *Campylobacter jejuni* ATCC 33560 and *Listeria monocytogenes* ATCC19112 were purchased from the American Type Culture Collection (Rockville, MD, USA). *Salmonella* Parathyphi A NCTC13 and *Yersinia enterocolitica* NCTC 11175 were obtained from the National Collection of Type Cultures (London, UK).

Preparation of Propolis Extracts

Propolis was obtained by *Apis mellifera* from pine, eucalyptus, orange and lemon trees in April 2018, Adana, Turkey. Propolis was collected using plastic traps which placed on top of hive and then stored in the freezer overnight for hardening of the samples. For extraction of propolis, crude propolis was grounded into powder. Ethanol (70%) or water (100%) were added and then, they placed in daily shakable containers for 48 h. Solutions of propolis were prepared aseptically and protected from bright light to prevent photo degradation. They were stored in a dark place at 4°C until analysis. Forty g of each extracts were used and their antimicrobial activity tested.

Total Phenol Content

Total phenol content of propolis was determined using a spectrophotometric Folin-Ciocalteau method [24] with minor modifications. The samples were prepared in triplicate for each analysis and the mean value of absorbance was measured. The unit was given as mg gallic acid equivalent (GAE)/g of honey sample.

Antimicrobial Activity of Extracts

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC): Clinical and Laboratory Standards Institute's methods [25] were applied for determination of MIC and MBC. One mL of plant extract (with stock solution of 50 mg/mL) was added to the first tube in each series and subsequently two-fold serially diluted with Mueller Hinton Broth (MHB). The inoculum suspension (1 mL) of each bacterial strain (10^6 cfu/mL) was then added in each tube containing plant extract and MHB. The final concentrations of the extract were 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19 mg/mL. Each tube was evaluated for bacterial growth and compared to the control. As a positive

control, a tube containing MHB and bacterial suspension without extracts was used. As a negative control, a tube not having MHB was used. The tubes were incubated at 35°C for 18-24 h after which the MIC was recorded. MIC was defined as the lowest concentration inhibiting bacterial growth MBC was determined by sub-culturing the contents of tubes of MIC showing no growth.

Disc Diffusion Method: The antimicrobial activity of extracts was determined using the disc diffusion method [26] with minor modifications. Mueller Hinton Agar was employed as the standard test medium for bacteria. The agar plate was spread with the inoculum having 10^8 CFU/mL pathogenic bacteria. Fifty microliters of diluted (50 mg/mL) and undiluted (100%) extracts were pipetted on sterile filter paper discs (diameter 6 mm). After incubation at 37°C for 18-24 h for bacteria, diameters (mm) of the zones of bacterial inhibition minus the disc diameter were determined. Each test was carried out in triplicate. Ethanolic alcohol solution were also tested as control. Antibiotics of tetracycline, streptomycin and vancomycin with positive responses were utilized as the control for the plates.

Culture Conditions and BAs Analysis: The production of BAs from all food borne pathogens in this work was monitored using histidine decarboxylase broth (HDB) containing 1 g peptone, 0.5 g Lab-Lemco powder (Oxoid CM0017, Hampshire, England), 2.5 g NaCl (Merck 1.06404.1000, Darmstadt, Germany), 4.01 g L-histidine HCl (Sigma H8125, Steinheim, Germany) and 2.5 mg pyridoxal-HCl (Sigma P9130, Steinheim, Germany) in 500 mL distilled water and, the pH was adjusted according to their optimum growth pH with 1 M KOH (Riedel-deHaen 06005, Seelze, Germany) or 6% TCA (Riedel-deHaen 27242, Seelze, Germany). After that HDB was pipetted in 10 mL bottles and then autoclaved at 121°C in 15 min prior to use. Extraction process and derivatisation of biogenic amines were performed according to the method of Kuley and Ozogul [27]. The confirmation of biogenic amine production was carried out using a rapid HPLC method [28]. For ammonia and trimethylamine (TMA) analysis, same analytic method was conducted.

Monitoring Bacterial Growth in HDB: Triplicate samples were taken to estimate total viable counts in HDB. Total viable bacteria were grown on plate count agar (Fluka 70152; Steinheim, Switzerland) as a spread plate using 0.1 mL of appropriately diluted samples for 2 days at 30°C.

Statistical Analysis

To find the average value and standard deviation, the data obtained from the three samples for each treatment was used. The between-group differences were analysed using one way ANOVA and its post-hoc analyses Duncan's multiple comparison test with SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL. USA).

RESULT

Total phenol contents of water and ethanolic extract of propolis were 20.85±0.74 and 53.41±1.03 mg gallic acid equivalent (GAE)/g, respectively.

Table 1 shows MIC and MBC of both propolis extracts against food-borne pathogens. MIC of ethanolic extracts was in range from 0.78 mg/mL for *Staph. aureus* to 12.5 mg/mL for *E. faecalis* and *S.* Paratyphi A. *Y. enterocolitica* and *K. pneumoniae* had similar MIC for ethanolic extracts of propolis with value of 3.12 mg/mL. MIC of water extract of propolis was between 3.12 mg/mL for *L. monocytogenes* and 50 mg/mL for *E. faecalis*. Water extracts of propolis also showed similar MIC (25 mg/mL) for *C. jejuni, Y. enterocolitica* and *S.* Parathyphi A. The respective MBC of ethanolic and water extracts of propolis was more than 6.25 and 12.5 mg/mL against food-borne pathogens. MBC for both extracts against *E. faecalis* and *S.* Paratyphi A was higher than 50 mg/mL.

Inhibition zones of Gram-negative and positive food-borne pathogens against diluted and undiluted propolis extracts and control antibiotics were given in *Table 2*. Significant differences were observed in inhibition zones of bacteria among groups ($P<0.05$). Surprisingly, undiluted water extract of propolis (100%) was the most effective on growth inhibition of *Staph. aureus* (29.5 mm) and *K. pneumoniae* (26.5 mm), compared to control antibiotics and ethanolic extracts of propolis. Undiluted ethanolic extracts inhibited *E. faecalis* growth stronger (26 mm) than the used antibiotics (<21.5 mm).

Table 1. *Minimum inhibition and bactericide concentration (mg/mL) of propolis extracts against Gram-negative and positive food-borne pathogen*

Bacterial Strains		Ethanolic Extracts of Propolis		Water Extracts of Propolis	
		MIC	MBC	MIC	MBC
Gram-positive bacteria	*L. monocytogenes*	1.56	6.25	3.12	25
	E. faecalis	12.5	>50	50	>50
	S. aureus	0.78	6.25	6.25	12.5
Gram-negative bacteria	*Y. enterocolitica*	3.12	50	25	>50
	C. jejuni	6.25	25	25	>50
	K. pneumoniae	3.12	6.25	12.5	50
	S. Parathyphi A	12.5	>50	25	>50

Table 2. *Inhibition zones (mm) of food-borne pathogens against propolis extracts and control*

Bacterial Strains		Water Extracts of Propolis		Ethanolic Extracts of Propolis		Control Antibiotics		
		50 mg/mL	100%	50 mg/mL	100%	TET	VAN	STREP
Gram-positive bacteria	*L. monocytogenes*	1.25*f (0.20)	11.00d (0.71)	0.00g (0.00)	3.05e (0.21)	20.75a (0.96)	19.50b (1.00)	12.50c (0.58)
	E. faecalis	1.25f (0.10)	9.00e (0.50)	13.25d (1.26)	26.00a (1.41)	16.00c (0.82)	21.50b (0.58)	0.00g (0.00)
	Staph. aureus	9.75^{e} (0.96)	29.5^{a} (2.12)	5.67^{f} (0.58)	9.00^{e} (0.71)	20.50^{c} (1.73)	24.75^{ba} (0.96)	14.00^{d} (0.82)
Gram-negative bacteria	*S.* Parathyphi A	2.69^{f} (0.24)	10.50^{c} (0.71)	8.25^{d} (0.50)	13.00^{b} (0.71)	21.75^{a} (0.96)	23.00^{a} (1.73)	5.33^{e} (0.29)
	K. pneumoniae	10.00^{d} (0.82)	26.50^{a} (0.71)	11.50^{d} (0.58)	20.50^{b} (0.71)	19.75b (1.50)	21.75b (2.06)	15.25c (0.50)
	C. jejuni	3.75d (0.08)	11.00c (0.42)	11.50c (0.08)	30.50a (0.42)	31.75a (1.71)	31.75a (0.96)	19.50b (1.00)
	Y. enterocolitica	1.25f (0.10)	11.50c (0.71)	5.00e (0.14)	10.00d (0.57)	26.75a (0.96)	23.50b (1.29)	0.00f (0.00)

** Data are expressed as mean value of three samples, Mean value (Standard deviation); $^{a-g}$ Indicate significant differences (P<0.05) between control and treated group in a column;* ***TET:*** *Tetracycline,* ***VAN:*** *Vancomycin,* ***STREP:*** *Streptomycin*

Table 3. *Bacterial growth in histidine decarboxylase broth with or without propolis extracts (log cfu/mL)*

Bacterial Strains		Control	Ethanolic Extracts of Propolis	Water Extracts of Propolis
Gram-positive bacteria	*L. monocytogenes*	8.77±0.14*^{a}	8.16±0.10^{b}	7.68±0.02^{c}
	E. faecalis	8.54±0.14^{a}	7.81±0.05^{b}	7.85±0.27^{b}
	Staph. aureus	8.65±0.06^{a}	8.15±0.04^{b}	8.29±0.11^{b}
Gram-negative bacteria	*S.* Parathyphi A	8.61±0.01^{a}	7.52±0.12^{b}	6.60±0.26^{c}
	K. pneumoniae	8.28±0.05^{a}	7.26±0.25^{b}	8.05±0.02^{a}
	C. jejuni	8.72±0.03^{a}	7.86±0.06^{b}	7.83±0.26^{b}
	Y. enterocolitica	8.36±0.16^{a}	7.71±0.03^{b}	7.72±0.03^{b}

** Data are expressed as mean value of three samples, Mean value±Standard deviation; $^{a-c}$ Indicate significant differences (P<0.05) between control and treated group in a column*

Table 3 shows bacterial growth in HDB in the absence and presence of propolis extracts. Microbial loads in control groups were in range 8.28 log cfu/g for *K. pneumoniae* to 8.77 log cfu/g for *L. monocytogenes*. Presence of propolis in HDB had significant effect on reducing bacterial growth (P<0.05). Apart from S. Parathyphi A, *K. pneumoniae* and *L. monocytogenes*, statistically no differences in bacterial load among ethanolic or water extracts of propolis groups were observed. In the presence of water extract in HDB, *S.* Paratyphi A and *L. monocytogenes* had the lowest bacterial growth, with corresponding value of 6.60 and 7.68 log cfu/g.

Ammonia and biogenic amine production by Gram positive and negative food-borne pathogens in the absence and presence of propolis extracts were given in *Table 4* and *Table 5*, respectively. Ammonia produced more than 220 mg/L by food-borne pathogens in HDB. Water and ethanolic extracts showed similar effects on ammonia production by Gram-negative bacteria. Significant differences in biogenic amine production by bacteria were observed among the groups (P<0.05). The effect of propolis on biogenic amine production showed a discrepancy depending on the bacterial strains, specific amine and extract type. Moreover, biogenic amine production by bacteria were not well correlated with bacterial load in the broth medium.

Putrescine production by food-borne pathogens was in range from 10.80 mg/L by *S.* Paratyphi A to 38.23 mg/L by *Y. enterocolitica*. Cadaverine produced more than 3.5 mg/L by bacteria. Although presence of propolis extracts in the medium did not affect cadaverine production by *E. faecalis* and *S.* Paratyphi A, suppressive effect on cadaverine production was noticed by water extracts of propolis on Gram-positive bacteria and ethanolic extracts on Gram-negative bacteria.

Spermidine and spermine are formed from putrescine. Spermidine and spermine production were the highest by *K. pneumoniae* (40.48 mg/L) and *C. jejuni* (26.91 mg/L), respectively. Spermidine and spermine production by most of bacteria were considerably inhibited by propolis extracts, mainly ethanolic extracts.

Histamine production by Gram-positive food-borne patho-

Table 4. *Ammonia and biogenic amine production by Gram-positive food borne pathogen in the absence or presence of propolis extracts (mg/L)*

Bacteria	Group	AMN	PUT	CAD	SPD	TRP	PHEN	SPN	HIS	SER	TYR	TMA	DOP	AGM
LM	C	389.76±19.78*a	30.45±2.08b	3.77±0.03b	18.84±1.30a	2.60±0.14a	0.85±0.01b	16.19±0.81a	0.85±0.05b	3.12±0.02a	69.06±4.32a	17.47±1.20a	20.56±0.54a	24.72±2.42a
	PE	61.44±8.37c	2.21±0.05c	6.04±0.29a	0.00±0.00c	0.00±0.00b	1.20±0.10b	0.00±0.00b	0.16±0.01c	0.75±1.06b	0.38±0.03b	15.50±1.41ab	20.38±1.00a	6.24±0.10b
	PW	152.82±17.29b	46.53±3.80a	1.40±0.07c	4.15±0.35b	0.00±0.00b	2.96±0.30a	0.00±0.00b	1.43±0.11a	3.09±0.03a	2.05±2.22b	12.73±0.17b	15.38±0.18b	2.20±0.11b
EF	C	530.56±28.95a	11.00±0.82b	11.84±0.55a	14.70±0.57a	1.86±0.16b	1.20±0.10a	10.07±0.16a	1.16±0.01b	31.03±1.15b	908.69±15.09a	67.24±1.03a	78.65±7.78a	16.89±0.94a
	PE	74.65±2.10b	3.50±0.24c	10.46±0.44a	0.00±0.00c	0.00±0.00b	0.99±0.01b	4.44±0.15c	0.58±0.00c	1.45±0.14c	3.15±0.08c	2.32±0.20c	50.53±3.27b	10.82±0.70b
	PW	86.55±8.00b	34.06±0.77a	10.02±0.94a	2.17±0.13b	3.40±0.11a	0.47±0.04c	5.46±0.30b	1.67±0.05a	185.61±14.81a	520.40±29.23b	46.79±5.50b	2.77±0.02c	6.60±0.07c
SA	C	489.41±19.62a	22.74±1.78b	10.13±0.33a	7.69±0.32a	3.49±0.33a	0.00±0.00c	15.03±0.96a	0.19±0.01a	1.07±0.07c	300.02±4.33a	9.21±0.10a	14.33±0.12b	7.59±0.36a
	PE	222.07±29.92c	26.87±0.48b	4.10±5.16b	3.93±0.56ab	1.22±0.04c	13.47±1.20a	0.00±0.00b	0.97±0.58a	16.86±0.19b	2.95±0.44c	9.11±0.13a	99.77±9.50a	7.98±0.02a
	PW	342.90±16.13b	34.40±2.08a	5.50±0.00b	5.95±0.59a	2.48±0.00b	4.40±0.15b	0.60±0.04b	0.45±0.03a	22.25±0.60a	86.39±3.88b	9.89±0.63a	110.45±6.87a	7.06±0.73a

** Data are expressed as mean value of three samples, Mean value±Standard deviation; [a-b] Indicate significant differences (P<0.05) between control and treated group in a row; **LM:** Listeria monocytogenes; **EF:** Enterococcus faecalis; **SA:** Staphylococcus aureus; **C:** Control group without propolis extract addition; **PE:** Group treated with ethanolic extract of propolis; **PW:** Group treated with water extract of propolis; **AMN:** Ammonia; **PUT:** Putrescine; **CAD:** Cadaverine; **SPD:** Spermidine; **TRP:** Tryptamine; **PHEN:** 2-phenylethyl amine; **SPN:** Spermine; **HIS:** Histamine; **SER:** Serotonin; **TYR:** Tyramine; **TMA:** Trimethylamine; **DOP:** Dopamine; **AGM:** Agmatine*

Table 5. *Ammonia and biogenic amine production by Gram-negative food borne pathogen in the absence or presence of propolis extracts (mg/L)*

Bacteria	Group	AMN	PUT	CAD	SPD	TRP	PHEN	SPN	HIS	SER	TYR	TMA	DOP	AGM
YE	C	417.35±23.67a*	38.23±9.91a	9.43±0.69b	17.51±1.27a	0.00±0.00c	5.19±0.13a	3.98±0.33b	30.97±1.64a	1.01±0.00b	1.34±0.02b	14.28±0.26a	123.55±12.84a	6.80±0.08b
	PE	91.17±7.03b	9.93±0.89b	5.71±0.59c	0.00±0.00c	1.06±0.00a	3.14±0.08c	1.44±0.10c	0.38±0.02b	2.09±0.13a	0.87±0.05b	14.87±0.35a	126.05±11.27a	8.27±0.54a
	PW	69.44±8.67b	43.25±3.23a	23.39±0.07a	5.85±0.49b	0.44±0.02b	4.41±0.12b	5.28±0.40a	0.44±0.02b	2.47±0.11a	378.16±37.4a3	7.69±0.13b	14.71±0.54b	2.32±0.08c
CJ	C	221.32±18.23a	30.59±0.88b	10.25±0.95b	9.92±0.29a	2.75±0.05a	0.00±0.00c	26.91±2.30a	2.76±0.20a	4.15±0.73c	750.87±42.81a	25.68±0.89c	118.24±6.55c	16.30±1.09b
	PE	79.21±1.56b	10.31±0.06c	10.15±0.49b	0.00±0.00b	2.21±0.12b	1.09±0.05b	0.00±0.00c	0.43±0.05c	21.73±0.66b	2.40±0.01b	30.92±0.45b	139.82±6.67b	10.99±0.06c
	PW	89.06±0.81b	38.11±2.81a	18.06±0.49a	0.00±0.00b	1.55±0.13c	6.53±0.04a	21.15±1.91b	1.35±0.04b	31.29±1.47a	667.90±52.39a	37.19±1.18a	165.77±1.41a	25.55±1.05a
KP	C	828.45±81.73a	13.33±0.81b	17.68±0.14b	40.48±3.07a	3.50±0.16a	0.00±0.00c	17.13±1.75a	1.89±0.13a	6.89±0.17a	886.44±59.49a	32.14±2.03a	165.69±14.58a	19.65±1.50a
	PE	130.62±4.76b	0.00±0.00c	8.89±0.29c	0.00±0.00b	0.00±0.00b	19.36±0.99a	0.00±0.00b	0.51±0.03b	1.06±0.13c	1.07±0.02c	1.24±0.11c	8.05±0.58c	0.19±0.01c
	PW	123.13±9.33b	46.17±3.01a	25.95±0.37a	2.75±0.07b	0.00±0.00b	1.91±0.06b	0.00±0.00b	0.58±0.08b	4.15±0.11b	321.23±2.12b	11.42±0.06b	127.81±5.15b	11.57±0.91b
SP	C	513.38±43.60a	10.80±1.04b	13.57±0.84a	19.20±1.17a	1.37±0.10a	0.00±0.00c	11.52±0.61a	0.79±0.05a	3.61±0.09b	832.12±38.19a	33.85±2.34a	114.52±4.51a	13.30±1.27a
	PE	114.05±10.05b	2.52±0.02c	11.66±0.62a	2.33±0.12b	0.18±0.01b	0.69±0.08b	6.38±0.23b	0.56±0.08b	4.86±0.59a	15.37±0.55c	3.89±0.21c	35.09±2.42b	15.72±0.65a
	PW	149.69±33.86b	25.91±1.63a	12.46±0.05a	1.18±0.10b	0.19±0.02b	2.76±0.01a	0.00±0.00c	0.79±0.03a	5.03±0.23a	343.41±11.18b	16.73±0.27b	104.79±7.69a	14.19±0.99a

** Data are expressed as mean value of three samples, Mean value±Standard deviation; [a-c] Indicate significant differences (P<0.05) between control and treated group in a row; **YE:** Yersinia enterocolitica; **CJ:** Campylobacter jejuni; **KP:** Klebsiella pneumoniae; **SP:** Salmonella Paratyphi A; **C:** Control group without propolis extract addition; **PE:** Group treated with ethanolic extract of propolis; **PW:** Group treated with water extract of propolis; **AMN:** Ammonia; **PUT:** Putrescine; **CAD:** Cadaverine; **SPD:** Spermidine; **TRP:** Tryptamine; **PHEN:** 2-phenylethyl amine; **SPN;** Spermine; **HIS:** Histamine; **SER:** Serotonin; **TYR:** Tyramine; **TMA:** Trimethylamine; **DOP:** Dopamine; **AGM:** Agmatine*

gens was below 2 mg/L. Histamine production by *L. monocytogenes* and *E. faecalis* was the highest in the presence of water extracts of propolis and the lowest in the presence of ethanolic extracts of propolis. However, both propolis extracts generally resulted in significantly lower histamine accumulation by Gram-negative bacteria.

Tyramine and dopamine were one of the mostly produced amines by food-borne pathogens (>910 vs. 114 mg/L). Tyramine production by bacteria was generally suppressed by addition of propolis extracts (P<0.05). Trimethylamine (TMA) production varied from 9.21 mg/L for *Staph. aureus* to 67.24 mg/L for *E. faecalis*. TMA formation was generally inhibited in the presence of both propolis extracts. Among food borne pathogens, *E. faecalis* produced the highest amount of serotonin. Serotonin production by most of food-borne pathogens was stimulated by water extract of propolis. However, ethanolic extracts of propolis induced lower serotonin accumulation by *E. faecalis* and *L. monocytogenes*.

Agmatine production was the highest by *L. monocytogenes*, with value 24.72 mg/L. Although the effect of water and ethanolic extract of propolis on agmatine production by *Y. enterocolitica* and *C. jejuni* varied, they had significant effect on reducing agmatine production by *K. pneumoniae*.

DISCUSSION

In this study, the impacts of ethanolic and water extracts of propolis on growth of common food-borne pathogens and their biogenic amine production were evaluated. The finding of this study showed that total phenol content of ethanolic extract of propolis is two-fold higher than that of water extract. Ethanol has been proposed as a good solvent for polyphenol extraction and is known as safe for human consumption. Similarly, Ramanauskiene et al.[29] studied quality and antimicrobial activity of Lithuanian propolis prepared by different solutions (2.5%, 5%, and 10% propolis) and solvents (purified water, 70% v/v ethanol, 96.3% v/v ethanol, propylene glycol). They found the highest content of phenolic compounds in increased propolis solutions and propolis extracts, whilst the water extracted had the lowest amount of phenolic compounds from crude propolis. Sun et al.[30] indicated that phenolic compounds and antioxidant properties of Beijing propolis extracts were significantly dependent on the concentration of ethanol/water solvents and the highest extraction yield

and the strongest antioxidant properties was achieved by 75 wt.% ethanol/water solvent. The ethanol/water content and the propolis concentration were also found to correlate with the composition of phenolic compounds and flavonoids [31]. Chemical composition of propolis is also highly variable depending on the collection site, floral composition and climate [23]. Propolis contains a wide variety of polyphenolic compounds with antimicrobial activity, especially flavonoids, followed by aromatic acids, phenol acid esters, triterpenes, lignans, etc. [6,32]. In the current study, antimicrobial activity of propolis also varied depending on propolis concentration. The application of undiluted propolis extracts (100%) showed higher antimicrobial activity against both Gram-negative and positive bacteria than diluted extracts (50 mg/mL). Unlike the results of this study, Hazem et al.[33] reported the higher antimicrobial activity of the diluted aqueous and alcoholic solutions of propolis extracts. This may be due to differences in chemical properties of propolis extracts used as well as in concentrations of extracts used in the experiment. Diluted water extracts of propolis had the poorest effect on Gram-positive *E. faecalis* and Gram-negative *Y. enterocolitica* and *S.* Paratyphi A. Regardless of the dose used, ethanolic extracts of propolis seemed to more active against *S.* Paratyphi A, *C. jejuni* and *E. faecalis* than that of water extracts. Moreover, in comparison to ethanolic extracts, water extracts of propolis at both doses were more effective against Gram-positive *L. monocytogenes* and *Staph. aureus*. The biological effect of the main constituents found in water extracts of propolis is greater than that of the ethanol extracts [34].

Moreover, ethanolic extracts of propolis had lower MIC and MBC than that of water extract. This can be explained by water extraction of propolis resulted in a product containing less extracted compounds [35]. Kubiliene et al.[36] demonstrated propolis extracts made in pure water or oil only at room temperature, contained more than 5-10-fold lower amount of phenolic compounds, and exerted no activity. Erturk et al.[37] found that ethanol extract of propolis had high antimicrobial activity against *Streptococcus mutans*, *L. monocytogenes, Micrococcus luteus, Bacillus licheniformis* and *Candida albicans*, whereas water extracts of propolis was not effective against all pathogens except for *S. mutans*. Water extracted propolis solutions did not inhibit the growth of the studied microorganisms [29].

Staph. aureus and *L. monocytogenes* were found as the most sensitive bacteria, although *E. faecalis* was the most resistant against both propolis extracts. Stepanović et al.[17] found that *E. faecalis* was the most resistant Gram-positive bacterium, *Salmonella* spp. the most resistant Gram-negative bacteria against ethanolic extracts of propolis from different regions of Serbia, which is consistent with this study results. However, Ramanauskiene et al.[29] reported that compared to Gram-positive bacteria Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa,* and *Proteus mirabilis* were more sensitive to propolis ethanol extract, and water extracted propolis solutions did not inhibit the growth of the studied microorganisms. The variation of the antibacterial activity of propolis is connected with the chemical composition of propolis obtained from different areas, concentration of propolis extract and extraction methods [38,39]. In the current study, both propolis extract exerted a stronger inhibitory effect against Gram-positive bacteria apart from *E. faecalis* than Gram-negative bacteria. This is in agreement with published data showing higher antimicrobial activity of propolis extracts against Gram-positive bacteria [40]. This effect may be explained by the structural differences between Gram-negative and Gram-positive bacterial cell wall [41].

Tyramine, dopamine, agmatine, spermine and putrescine were reported as the main amines produced by food-borne pathogens in tyrosine decarboxylase broth [22]. Similarly, food-borne pathogens produced all biogenic amine tested, mainly tyramine, dopamine and putrescine as well as ammonia. Among Gram-positive bacteria, the highest ammonia production was observed for *E. faecalis*, whilst *K. pneumoniae* was main Gram-negative bacteria produced the highest level of ammonia with value of 828.45 mg/L. This is consistent with the results of Kuley and Ozogul [27]. Propolis extracts significantly inhibited ammonia production by all bacteria tested. Ethanolic extracts had considerably higher inhibition effect on reducing ammonia production by Gram-positive *L. monocytogenes* and *Staph. aureus* than that of water extracts of propolis.

Putrescine is a commonly occurring biogenic amine in food mainly due to the bacterial metabolism of the Gram-negative as well as Gram-positive bacteria and is potentially carcinogenic [42]. Conversion of ornithine into putrescine by *S.* Parathyphi A, *L. monocytogenes* and *Staph. aureus* was reported as above 75 mg/L [43]. In the current study, these bacteria formed putrescine below 31 mg/L. Apart from *Staph. aureus* and *Y. enterocolitica*, ethanolic extracts of propolis significantly induced lower putrescine accumulation by bacteria, whilst water extract stimulated putrescine production by bacteria. The highest inhibitory effect of ethanolic extracts on putrescine production was observed for *L. monocytogenes* and *K. pneumoniae* with 13 fold-lower putrescine production.

Histamine in foods occurs because of the decarboxylation of its precursor amino acid, histidine, by the action of the bacterial enzyme L-histidine decarboxylase [44]. Gram-negative bacteria accumulated histamine in range from 0.79 mg/L for *S.* Paratyphi A to 30.97 mg/L by *Y. enterocolitica*. *K. pneumoniae* was the most prolific histamine producer [45]. However, in the current study, *Y. enterocolitica* had a higher ability to produce histamine than that of *K. pneumoniae*. Among Gram-positive bacteria, *Staph. aureus* was not affected from presence of propolis extract on the production of histamine. Water or ethanolic extracts of propolis induced about 81-fold lower histamine accumulation by

Y. enterocolitica. The presence of extract did not have any effect on production of histamine by *Staph. aureus*.

The availability of free 5-hydoxytryptophan and tyrosine in the medium may result in the production of serotonin and dopamine [46]. *E. faecalis* and *K. pneumoniae* had the highest ability to produce serotonin (31.03 mg/L) and dopamine (165.69 mg/L) in HDB, respectively. Serotonin production by *E. faecalis* and *Staph. aureus* was significantly stimulated by water extract of propolis. BAs is formed by bacterial decarboxylation of free amino acids. Various studies showed that propolis contained various free amino acids including histidine, tyrosine, arginine, lysine, phenylalanine and tryptophan [47]. This stimulation effects may be attributed to chemical content of propolis. Serotonin production by Gram-negative bacteria except for *K. pneumoniae* was also increased by addition of both extracts. Dopamine plays an essential role in humans for the coordination of body movements, motivation, and reward [48]. The extract application generally tended to reduce dopamine production by bacteria apart from *Staph. aureus* and *C. jejuni* that their productions increased considerably with addition of extracts. Propolis ethanolic extracts did not affect dopamine production by *L. monocytogenes* and *Y. enterocolitica*.

Bover-Cid and Holzapfel [49] reported that *E. faecalis* accumulated tyramine. Similarly, *E. faecalis* was the main tyramine producer (908.69 mg/L) among Gram-positive bacteria, whereas the most Gram-negative bacteria produced tyramine more than 750 mg/L. Propolis ethanolic extracts showed significant inhibition effect on tyramine production by all bacteria tested which induced more than 55-fold lower tyramine accumulation. Water extract of propolis also suppressed tyramine production by all Gram-positive bacteria ($P<0.05$), although inhibition effect on tyramine production by Gram-negative bacteria was only found for *K. pneumoniae* and *S.* Paratyphi A.

Arginine is converted to agmatine by arginine decarboxylase and further converted into putrescine by agmatine deiminase system [46]. *L. monocytogenes* accumulated the highest level of agmatine compared to other food-borne pathogens. Among Gram-positive bacteria, agmatine production noticeably reduced in the presence of both propolis extract ($P<0.05$), whilst these extracts did not change agmatine production by *Staph. aureus*.

In conclusion, although ethanolic extract contained more total phenolic compounds, the effects of the extracts on bacteria were variable depending on activity test, concentration used and specific amine. Both propolis extracts generally showed a significantly stronger growth inhibitory effect against Gram-positive bacteria than Gram-negative bacteria. *Staph. aureus* and *L. monocytogenes* were found as the most sensitive bacteria, although *E. faecalis* was the most resistant bacteria against both propolis extracts. The application of high concentration of propolis extracts showed higher antimicrobial activity against both Gram-negative and positive bacteria than that of low dose of extracts. Undiluted water extract of propolis was also found more effective on growth inhibition of *Staph. aureus* and *K. pneumoniae*, compared to tetracycline, vancomycin and streptomycin antibiotics. As far as we know, no studies have been conducted assessing the effects of propolis on bacterial biogenic amine production. The study results revealed that histamine production by Gram-negative bacteria significantly suppressed, but their effects on Gram-positive bacteria were inconstant. Tyramine formation by Gram positive and negative bacteria was generally inhibited in the presence of propolis extracts. Although it exerted variability even among Gram-positive or negative bacteria, it was suggested that it could be used as antimicrobial agent as it usually inhibits biogenic amines such as tyramine and histamine which was toxically important. Moreover, serotonin production by bacteria was generally stimulated by both propolis extracts, mainly water extract. Serotonin is an important chemical and neurotransmitter in the human body, which is best known for its positive effect on mood. This positive aspect of propolis has not been emphasized in studies conducted so far. Detailed studies are also needed to understand the exact mechanism of these extracts on biogenic amine production.

REFERENCES

1. Tzima K, Makris D, Nikiforidis CV, Mourtzinos I: Potential use of rosemary, propolis and thyme as natural food preservatives. *J Nutr Health*, 1 (1): 6, 2015.

2. Mishima S, Inoh Y, Narita Y, Ohta S, Sakamoto T, Araki Y, Nozawa Y: Identification of caffeoylquinic acid derivatives from Brazilian propolis as constituents involved in induction of granulocytic differentiation of HL-60 cells. *Bioorg Med Chem*, 13 (20): 5814-5818, 2005. DOI: 10.1016/j.bmc.2005.05.044

3. Burdock GA: Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem Toxicol,* 36 (4): 347–363, 1998. DOI: 10.1016/S0278-6915(97)00145-2

4. Casquete R, Castro SM, Jácome S, Teixeira P: Antimicrobial activity of ethanolic extract of propolis in "Alheira", a fermented meat sausage. *Cogent Food Agri*, 2 (1): 1-7, 2016. DOI: 10.1080/23311932.2015.1125774

5. Chen YW, Ye SR, Ting C, Yu YH: Antibacterial activity of propolins from Taiwanese green propolis. *J Food Drug Anal*, 26 (2): 761-768, 2018. DOI: 10.1016/j.jfda.2017.10.002

6. Machado CS, Mokochinski JB, de Lira TO, de Oliveira FDCE, Cardoso MV, Ferreira RG, Sawaya AC, Ferreira AG, Pessoa C, Cuesta-Rubio O, Monteiro MC, de Campos MS, Torres YR: Comparative study of chemical composition and biological activity of yellow, green, brown, and red Brazilian propolis. *Evid Based Complement Alternat Med*, 2016:6057650, 2016. DOI: 10.1155/2016/6057650

7. Zabaiou N, Fouache A, Trousson A, Baron S, Zellagui A, Lahouel M, Lobaccaro JMA: Biological properties of propolis extracts: Something new from an ancient product. *Chem Phys Lipids*, 207, 214-222, 2017. DOI: 10.1016/j.chemphyslip. 2017.04.005

8. Açıkgöz Z, Yücel B, Altan Ö: The effects of propolis supplementation on broiler performance and feed digestibility. *Arch Geflügelk*, 69 (3): 117-122, 2005.

9. Yücel B, Önenç A, Kaya A, Altan Ö: Effects of propolis administration on growth performance and neonatal diarrhea of calves. *SOJ Vet Sci*, 1 (1): 102-106, 2015. DOI: 10.15226/2381-2907/1/1/00102

10. Bankova V, Popova M, Trusheva B: New emerging fields of application

of propolis. *Maced J Chem Chem En*, 35 (1): 1-11, 2016. DOI: 10.20450/mjcce.2016.864

11. Koc AN, Silici S, Mutlu Sariguzel F, Sagdic O: Antifungal activity of propolis in four different fruit juices. *Food Technol Biotechnol*, 45 (1): 57-61, 2007.

12. Aly SA, Elewa NA: The effect of Egyptian honeybee propolis on the growth of *Aspergillus versicolor* and sterigmatocystin biosynthesis in Ras cheese. *J Dairy Sci*, 74 (1): 74-78, 2007. DOI: 10.1017/S002202990600207X

13. Vargas-Sánchez RD, Torrescano-Urrutia GR, Acedo-Félix E, Carvajal-Millán E, González-Córdova AF, Vallejo-Galland B, Sánchez-Escalante A: Antioxidant and antimicrobial activity of commercial propolis extract in beef patties. *J Food Sci*, 79 (8): C1499-C1504, 2014. DOI: 10.1111/1750-3841.12533

14. Nedji N, Loucif-Ayad W: Antimicrobial activity of Algerian propolis in foodborne pathogens and its quantitative chemical composition. *Asian Pac J Trop Dis*, 4 (6): 433-437, 2014. DOI: 10.1016/S2222-1808(14)60601-0

15. El-Mossalami HHA, Abdel-Hakeim YA: Using of propolis extract as a trial to extend the shelf-life and improving the quality criteria of fresh Egyptian sausage. *Assiut Vet Med J*, 59 (139): 23-33, 2013.

16. Castaldo S, Capasso F: Propolis, an old remedy used in modern medicine. *Fitoterapia*, 73, S1-S6, 2002. DOI: 10.1016/S0367-326X(02)00185-5

17. Stepanović S, Antić N, Dakić I, Švabić-Vlahović M: *In vitro* antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. *Microbiol Res*, 158 (4): 353-357, 2003. DOI: 10.1078/0944-5013-00215

18. Suzzi G, Torriani S: Biogenic amines in foods. *Front Microbiol*, 6, 472, 2015. DOI: 10.3389/fmicb.2015.00472

19. Prester L: Biogenic amines in fish, fish products and shellfish. A review. *Food Addit Contam Part A*, 28 (11): 1547-1560, 2011. DOI: 10.1080/19440049.2011.600728

20. Stratton JE, Hutkins RW, Taylor SL: Biogenic amines in cheese and other fermented foods: A review. *J Food Prot*, 54 (6): 460-470, 1991. DOI: 10.4315/0362-028X-54.6.460

21. Hao R, Liu Y, Sun L, Xia L, Jia H, Li Q, Pan J: Sodium alginate coating with plant extract affected microbial communities, biogenic amine formation and quality properties of abalone *(Haliotis discus hannai* Ino) during chill storage. *LWT-Food Sci Technol*, 81, 1-9, 2017. DOI: 10.1016/j.lwt.2017.03.031

22. Özogul F, Kaçar Ç, Kuley E: The impact of carvacrol on ammonia and biogenic amine production by common foodborne pathogens. *J Food Sci*, 80 (12): 2899-2903, 2015. DOI: 10.1111/1750-3841.13140

23. Gajger IT, Pavlović I, Bojić M, Kosalec I, Srečec S, Vlainić T, Vlainić J: The components responsible for the antimicrobial activity of propolis from Continental and Mediterranean regions in Croatia. *Czech J Food Sci*, 35 (5): 376-385, 2017. DOI: 10.17221/103/2017-CJFS

24. Singleton VL, OrthoferR, Lamuela-Raventos RM: Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Method Enzymol,* 299, 152-178, 1999. DOI: 10.1016/S0076-6879(99)99017-1

25. Clinical and Laboratory Standards Institute: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *CLSI Wayne PA*, 2008.

26. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH: Manual of clinical microbiology. 6th ed., 1482, American Society of Microbiology Press, Washington DC, 1995.

27. Kuley E, Özogul **F:** Synergistic and antagonistic effect of lactic acid bacteria on tyramine production by food-borne pathogenic bacteria in tyrosine decarboxylase broth. *Food Chem,* 127 (3): 1163-1168, 2011. DOI: 10.1016/j.foodchem.2011.01.118

28. Özogul F: Production of biogenic amines by *Morganella morganii, Klebsiella pneumoniae* and *Hafnia alvei* using a rapid HPLC method. *Eur Food Res Technol*, 219 (5): 465-469, 2004. DOI: 10.1007/s00217-004-0988-0

29. Ramanauskienė K, Inkėnienė AM, Petrikaitė V, Briedis V: Total phenolic content and antimicrobial activity of different lithuanian propolis solutions. *Evid Based Complement Alternat Med*, 2013:842985, 2013. DOI: 10.1155/2013/842985

30. Sun C, Wu Z, Wang Z, Zhang H: Effect of ethanol/water solvents on phenolic profiles and antioxidant properties of Beijing propolis extracts. *Evid Based Complement Alternat Med*, 2015:595393, 2015. DOI: 10.1155/2015/595393

31. Cottica SM, Sawaya AC, Eberlin MN, Franco SL, Zeoula LM, Visentainer JV: Antioxidant activity and composition of propolis obtained by different methods of extraction. *J Braz Chem Soc,* 22 (5): 929-935, 2011. DOI: 10.1590/S0103-50532011000500016

32. Popova M, Silici S, Kaftanoglu O, Bankova V: Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. *Phytomedicine*, 12 (3): 221–228, 2005. DOI: 10.1016/j.phymed.2003.09.007

33. Hazem A, Popescu CV, Crisan J, Popa M, Chifiriuc M, Pircalabioru GG, Lupuliasa D: Antibacterial efficiency of five propolis extracts on planktonic and adherent microbial strains. *Farmacia*, 65 (5): 813-818, 2017.

34. Nakajima Y, Shimazawa M, Mishima S, Hara H: Water extract of propolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. *Life Sci*, 80 (4): 370-377, 2007. DOI: 10.1016/j.lfs.2006.09.017

35. Park YK, Alencar SM, Aguiar CL: Botanical origin and chemical composition of Brazilian propolis. *J Agric Food Chem*, 50 (9): 2502–2506, 2002. DOI: 10.1021/jf011432b

36. Kubiliene L, Laugaliene V, Pavilonis A, Maruska A, Majiene D, Barcauskaite K, Savickas A: Alternative preparation of propolis extracts: Comparison of their composition and biological activities. *BMC Complement Altern Med*, 15 (1): 156, 2015. DOI: 10.1186/s12906-015-0677-5

37. Ertürk Ö, Çil E, Yoloğlu N, Yavuz C: An *in vitro* study on antimicrobial and antioxidant activity of propolis from Rize province of Turkey. *Mellifera*, 16 (1): 4-18, 2016.

38. Bankova V, Popova M, Bogdanov S, Sabatini AG: Chemical compositiosition of European propolis: Expected and unexpected results. *Z Naturforsch B Chem Sci*, 57 (5-6): 530-533, 2002.

39. Kim YH, Chung HJ: The effects of Korean propolis against foodborne pathogens and transmission electron microscopic examination *New Biotechnol*, 28 (6): 713-718, 2011. DOI: 10.1016/j.nbt.2010.12.006

40. Silva RPD, Machado BAS, de Abreu Barreto G, Costa SS, Andrade LN, Amaral RG, Umsza-Guez MA: Antioxidant, antimicrobial, antiparasitic, and cytotoxic properties of various Brazilian propolis extracts. *Plos One*, 12 (13): e0172585, 2017. DOI: 10.1371/journal.pone.0172585

41. Orsi RDO, Sforcin JM, Funari SRC, Fernandes JA, Bankova V: Synergistic effect of propolis and antibiotics on the *Salmonella typhi. Braz J Microbiol*, 37 (2): 108-112, 2006. DOI: 10.1590/S1517-83822006000200002

42. Wunderlichová L, Buňková L, Koutný M, Jančová P, Buňka F: Formation, degradation, and detoxification of putrescine by foodborne bacteria: A review. *Compr Rev Food Sci*, 13, 1012-1030, 2014. DOI: 10.1111/1541-4337.12099

43. Kuley E, Balikci E, Özogul İ, Cengiz D: Interaction between lactic acid bacteria and food-borne pathogens on putrescine production in ornithine-enriched broth. *J Food Sci Technol.*, 48 (2): 394-404, 2013. DOI: 10.1111/j.1365-2621.2012.03201.x

44. Comas-Basté O, Latorre-Moratalla ML, Sánchez-Pérez S, Veciana-Nogués MT, del Carmen Vidal-Carou M: Histamine and other biogenic amines in food. From scombroid poisoning to histamine intolerance. **In,** Proestos C (Ed): Biogenic Amines. 1-19, IntechOpen, Rijeka, 2019. DOI: 10.5772/ intechopen.84333

45. Emborg J, Dalgaard P: Formation of histamine and biogenic amines in cold smoked tuna: An investigation of psychrotolerant bacteria from samples implicated in cases of histamine fish poisoning. *J Food Prot*, 69 (4): 897-906, 2006. DOI: 10.4315/0362-028X-69.4.897

46. Udenfriend S, Titus E, Weissbach H, Peterson RE: Biogenesis and metabolism of 5-hydroxyindole compounds. *J Biol Chem*, 219 (1): 335-344, 1956.

47. Eroglu N, Akkus S, Yaman M, Asci B, Silici S: Amino acid and vitamin content of propolis collected by native caucasican honeybees. *J Apic Sci*, 60 (2): 101-110, 2016. DOI: 10.1515/jas-2016-0021

48. Mah JH, Hwang HJ: Effects of food additives on biogenic amine formation in *Myeolchi-jeot*, a salted and fermented anchovy *(Engraulis japonicus). Food Chem*, 114 (1): 168–173, 2009. DOI: 10.1016/j.foodchem.2008.09.035

49. Bover-Cid S, Holzapfel WH: Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int J Food Microbiol*, 53 (1): 33-41, 1999. DOI: 10.1016/S0168-1605(99)00152-X

Kafkas Univ Vet Fak Derg
26 (2): 201-207, 2020
DOI: 10.9775/kvfd.2019.22730

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Identification and Characterization of Immunogenic Genes from Genomic Expression Library of *Mycoplasma ovipneumoniae*

Mengfan QIAO [1,2,†,a] Cheng CHEN [1,†,b] Sufang YANG [3,†,c] Xingxing ZHANG [1,d] Lulu TIAN [1,e]
Haiting LU [1,f] Shasha GONG [1,g] Xuepeng CAI [4,h] Qingling MENG [1,i] Jun QIAO [1,j]

[†] *These authors contribute equally*
[1] College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832003, CHINA
[2] College of Biotechnology, Central South University, Changsha, Hunan, 410012, CHINA
[3] School of Biotechnology, Bayingol Vocational and Technical College, Korla, Xinjiang, 841000, CHINA
[4] State Key Lab of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu, 730046, CHINA
[a] ORCID: 0000-0002-5432-0988; [b] ORCID: 0000-0002-8187-7722; [c] ORCID: 0000-0002-7534-1745; [d] ORCID: 0000-0001-8085-154X
[e] ORCID: 0000-0001-5820-0337; [f] ORCID: 0000-0003-2748-4714; [g] ORCID: 0000-0003-0236-2323; [h] ORCID: 0000-0001-5725-1968
[i] ORCID: 0000-0002-1190-0114; [j] ORCID: 0000-0001-5210-4379

Article ID: KVFD-2019-22730 Received: 25.05.2019 Accepted: 06.10.2019 Published Online: 08.10.2019

How to Cite This Article

Qiao M,A Chen C, Yang S, Zhang X, Tian L, Lu H, Gong S, Cai X, Meng Q, Qiao J: Identification and characterization of immunogenic genes from genomic expression library of *Mycoplasma ovipneumoniae. Kafkas Univ Vet Fak Derg,* 26 (2): 201-207, 2020. DOI: 10.9775/kvfd.2019.22730

Abstract

Mycoplasma ovipneumoniae is an important pathogen causing respiratory disease in sheep. At present, the immune-associated antigens of *M. ovipneumoniae* are still unknown, which significantly limits the development of new vaccines for *M. ovipneumoniae.* In order to identify and characterize the immune-associated antigen genes, genomic expression library of *M. ovipneumoniae* was constructed and identified, from which positive clones were recognized and screened by positive serum against *M. ovipneumoniae.* Sequence analysis showed that these 10 clones contained 5 different genes encoding P97-like protein, P102-like protein, Translation initiation factor (IF-1), Methionine aminopeptidase (MAP) and P56 membrane protein, respectively. Three proteins including IF-1, MAP and P97-like protein were expressed in *E. coli* and used to immunize lambs to verify their immunogenicity, respectively. Animal immunization test confirmed that the novel protein MAP displayed a strong immunogenicity, while the immunogenicity of P97-like protein and IF-1 were relatively weak. The identification of immunogenic protein MAP provided a potentially valuable antigen candidate for the development of serological diagnostic method and subunit vaccine against *M. ovipneumoniae* infection.

Keywords: *Mycoplasma ovipneumoniae, Immune-associated antigen, Screening, Characterization*

Mycoplasma ovipneumoniae'nın Genomik İfade Kütüphanesinden İmmünojenik Genlerin Belirlenmesi ve Karakterizasyonu

Öz

Mycoplasma ovipneumoniae, koyunlarda solunum sistemi hastalığına neden olan önemli bir patojendir. Günümüzde, *M. ovipneumoniae*'nin immün ilişkili antijenleri hala bilinmemektedir ve bu durum *M. ovipneumoniae*'ya karşı yeni aşıların geliştirilmesini önemli ölçüde sınırlamaktadır. Çalışmada immün ilişkili antijen genlerinin tanımlanması ve karakterizasyonu amacıyla, *M. ovipneumoniae*'ye karşı pozitif klonlar pozitif serum tarafından belirlenip taranarak *M. ovipneumoniae*'nin genomik ekspresyon kütüphanesi oluşturuldu. Sekans analizi, bu 10 klonun, sırasıyla P97-benzeri protein, P102-benzeri protein, translasyon başlatma faktörü (IF-1), Metiyonin aminopeptidaz (MAP) ve P56 membran proteinini kodlayan 5 farklı gen içerdiğini gösterdi. Sırasıyla IF-1, MAP ve P97-benzeri proteini içeren üç protein, *E. coli*'de eksprese edildi ve immünojenisitelerini doğrulamak için kuzuların immünizasyonunda kullanıldı. Hayvan immünizasyon testi, yeni protein MAP'ın güçlü bir immünojenisite sergilediğini, P97 benzeri proteinin ve IF-1'in immünojenisitesinin nispeten zayıf olduğunu doğruladı. İmmünojenik protein MAP'ın tanımlanması, *M. ovipneumoniae* enfeksiyonuna karşı serolojik tanı yöntemi ve alt ünite aşı geliştirilmesi için değerli bir potansiyel antijen adayı elde edilmesini sağladı.

Anahtar sözcükler: *Mycoplasma ovipneumoniae, İmmün ilişkili antijen, Tarama, Karakterizasyon*

İletişim (Correspondence)
+86-993-2055036 Fax: +86-993-2055038
qj710625@shzu.edu.cn

INTRODUCTION

Mycoplasma ovipneumoniae is a respiratory pathogen causing interstitial pneumonia in sheep and goats [1-4], which is characterized by chronic non-progressive pneumonia with clinical manifestations of cough, wheezing, runny nose, anemia, weight loss and growth retardation [5,6]. The disease is widely prevalent in sheep farming countries and has caused tremendous economic losses. In recent years, with the rapid development of China's sheep industry, sheep pneumonia caused by *M. ovipneumoniae* has been widely prevalent in western regions of China including Gansu, Ningxia, Sichuan, and Xinjiang provinces. At present, it becomes one of the main infectious diseases in sheep [7,8]. More importantly, *M. ovipneumoniae* infection can also cause immunosuppression, leading to the increased susceptibility of sheep to other pathogens, e.g., *Pasteurella*, *Mannheimia haemolytica* and *Parainfluenza-3 virus* [9-13].

Due to the high nutritional requirements of *M. ovipneumoniae in vitro* cultivation, the production cost of conventional inactivated vaccine based on whole-cells is very high. Therefore, the development of new vaccine is of great significance for the control of *M. ovipneumoniae* infection. However, identification of microbial components that give rise to a protective immune response is the key to the development of subunit vaccine. Over the last decade, the genome of three *M. ovipneumoniae* epidemic strains (SC01 strain, NM2010 strain and France 14811) has been fully sequenced, however, the antigenic proteins have not been identified [14,15], which has greatly limited the development of serological diagnostics and subunit vaccines to prevent *M. ovipneumoniae* infection in sheep. Nowadays, construction of genomic expression library is considered as one of the effective strategies to identify genes encoding pathogens' immunogenic antigens. Using genomic expression library, antigenic proteins and virulence factors have been identified in *Mycoplasma mycoides* subsp. *mycoides* and *Campylobacter jejuni*, respectively [16,17]. Compared to other *Mycoplasma* spp. *(M. gallisepticum, M. hyopneumoniae, M. bovis and M. suis)* [18], antigenic proteins and virulence factors of *M. ovipneumoniae* was still not extensively studied.

The aim of this study is to identify and characterize immune-associated proteins encoded by the genome of *M. ovipneumoniae*. We constructed *M. ovipneumoniae* genomic expression library and identified clones that could be recognized by *M. ovipneumoniae* positive serum. The genes encoding these immune-associated proteins were then identified by sequencing, and the immunogenicity of recombinant proteins were further confirmed by animal immunization test.

MATERIAL and METHODS

Ethical Approval

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Shihezi University.

Culture of M. ovipneumoniae

Mycoplasma ovipneumoniae shz-1 strain was isolated from nasal secretions of a sheep infected with *M. ovipneumoniae* in a farm in Xinjiang province. *M. ovipneumoniae* was confirmed by biochemical and molecular methods. *M. ovipneumoniae* was stored in the Animal Disease Prevention and Control Laboratory of Xinjiang. Purified *M. ovipneumoniae* was inoculated onto Brain Heart Infusion (BHI) containing 15% (v/v) heat-inactivated horse serum (Biotopped, China), 0.004% (w/v) phenol red (Sigma, USA), and 25 μg/mL ampicillin (Omega, USA) and cultured at 37°C incubator with 5% CO_2 for 4 days.

Preparation of Positive Serum Against M. ovipneumoniae

Mycoplasma ovipneumoniae shz-1 strain was cultured to reach the concentration of 10^8 CCU/mL. Two 60-day-old lambs were infected with 500 μL culture (10^8 CCU/mL) of *M. ovipneumoniae* by intratracheal inoculation, respectively. After 4 weeks of inoculation, blood was collected, and antibody titer was determined by indirect hemagglutination assay (IHA) kit (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) as described previously [7]. The individual sera were combined and used for the screening of immunogenic genes from genomic expression library of *M. ovipneumoniae*.

Adsorption and Removing the Antibodies Against E. coli

The serum samples were mixed with the lysate of *E. coli* to remove the antibodies that have cross-reactions with *E. coli*. Briefly, saturated 100 mL *E. coli* BL21 culture was centrifuged at 12000 r/min and 4°C for 10 min. The bacteria pellet was resuspended in 10 mL PBS buffer. Following three times of freezing and thawing, bacteria were lysed by sonication for 6 times (10 sec for each time and 10 sec interval). Subsequently, 500 μL of sonication positive serum was mixed with the *E. coli* cell lysate for 2 h. The supernatant serum was collected after 10 min of centrifugation at 12.000 r/min, and 4°C. Western blot was performed to analyze the reaction between the *E. coli* whole cell lysate and the adsorbed serum. Qualified serum samples were stored at -20°C.

Generation of Genomic Expression Library of M. ovipneumoniae

Mycoplasma ovipneumoniae genomic DNA (gDNA) was extracted using Mini Extraction Kit (BIOMIGA, USA). The gDNA was digested by restriction enzyme *Sau*3A I (TaKaRa, Japan). The digested fragments were ligated to the expression vector pET28 (a/b/c) (Invitrogen, USA) that was pre-digested with *BamH* I (TaKaRa, Japan) and dephosphorylated, respectively. The ligation products were respectively transformed to *E. coli* DH5α competent cells (TaKaRa, Japan) and incubated at 37°C for 12 h. The plasmids were extracted and

then transferred into *E. coli* BL21 competent cells. A total of 100 colonies were randomly picked and the inserts were analyzed by PCR using T7 (5'-TAATACGACTCACTATAGGG-3') and T7ter (5'-TGCTAGTTATTGCTCAGCGG-3') primers [19].

Screening of Immunogenic Genes from Genomic Expression Library

Screening of library was performed as described previously [18]. Briefly, the library was diluted and plated on LB plate containing kanamycin (OMEGA, USA). After 8 h of incubation at 37°C, the clones were transferred onto a nitrocellulose membrane, which was subsequently placed on a new LB plate containing kanamycin and incubated for 2-3 h. Subsequently, the nitrocellulose membrane was plated on LB agarose plate containing 1 mM IPTG and cultured for 4-6 h at 37°C. The membrane was exposed to the chloroform steam for 15-20 min and air dried. The membrane was then placed in the lysis buffer (100 mM Tris-HCl pH7.8, 150 mM $MgCl_2$, 1.5% BSA, 1ug/ml pancreatic RNAase, 40 ug/mL lysozyme) (Sigma, USA) and incubated at room temperature for 14 h. The membrane was incubated with the *M. ovipneumoniae* positive serum (1:3000) at room temperature for 1 h followed by incubation at HRP-conjugated rabbit anti-sheep IgG (1:5000) (Bethyl, USA) for 1 h. Finally, the membrane was added with the TMB substrate and the positive clones were verified for several times.

Sequence Analysis of Genes Encoding Immunogenic Proteins

Plasmids were isolated from the positive clones that had reactions with *M. ovipneumoniae* positive serum, and each plasmid was sequenced 3 times. The sequence of recombinant plasmids from identical sequencing results was used for sequence analysis. Then, these sequences were blasted against NCBI database using blast X *(http://blast.ncbi.nlm.nih.gov/Blast.cgi)*. After verifying that the inserts were from *M. ovipneumoniae* genome, their molecular characteristics were analyzed.

Expression and Reactogenicity Analysis of Immunogenic Proteins

Based on the sequence of positive clones, specific primers *(Table 1)* were designed using Primer premier 5.0 software, respectively. Then, PCR-amplified fragments were cloned into the expression vector pET32a (+) to generate pET32a-IF-1, pET32a-MAP, pET32a-P97-like recombinant plasmids, respectively. These three recombinant plasmids were then transformed into BL21 competent cells. The transformants were cultured in LB medium containing the appropriate antibiotic, and protein expression was induced by adding a final concentration of 1 mM IPTG (TaKaRa, Japan) when OD_{600nm} of the culture reached 0.6 ~ 0.8. Culture was collected after 4, 6, 8 and 10 h of IPTG induction, respectively. Bacterial cells were harvested by centrifugation and used for SDS-PAGE analysis. *M. ovipneumoniae* positive serum was used as the primary antibody and HRP-conjugated rabbit anti-sheep antibody (Bethyl, USA) was used as secondary antibody for Western blot analysis. Protein was purified according the instruction of the Ni-NTA Purification System (Invitrogen, USA).

Immunogenicity Analysis of M. ovipneumoniae Immune-Related Antigens

Immunogenicity of purified proteins was evaluated in lambs. Briefly, a total of twenty 60-day-old lambs being antibody-negative were divided into 4 groups with 5 lambs in each group. Purified IF-1, MAP and P97-like protein were respectively mixed with Freund's complete adjuvant (Sigma, USA) to prepare different antigens with protein concentration at 0.05 μg/μL. PBS buffer was mixed with Freund's complete adjuvant at ratio of 1: 1 and was as the negative control. These antigens were intradermally injected at multiple sites at the dose of 500 μL/lambs, respectively. After 14 days, boost immunization was performed similarly. Seven days after the boost immunization, the blood samples from different groups were collected and sera were separated. IHA was preformed to determinate the specific antibody against *M. ovipneumoniae*.

RESULTS

Identification of M. ovipneumoniae Genomic Library

According to the *M. ovipneumoniae* genome size and an average of 1500 bp-size insert, 3.2×10^3 clones were needed to cover the entire genome. Because the DNA fragments were cloned into three different plasmids, and fragment ligation efficiency was over 90%, 10.436 clones from each vector were only needed to screen. In this study, a total of 2.5×10^4 clones were obtained, which is in line with the coverage requirement of genomic library.

Table 1. *List of primers used in this study*

Primer Name	Target Gene	Nucleotide Sequence (5'-3')	Product Size (bp)
P1	IF-1	GAATTCATGCAAAATTCTTCAAAAG	240
P2		CTCGAGTTATTTAAAGCGGTAAACA	
P3	MAP	GAATTCATGTCTCTAATTAAAACAGAAT	642
P4		CTCGAGTATTCGCGGAGAACTTT	
P5	P97-like	GAATTCATGAGTAAACCTAAACAAAAATC	714
P6		CTCGAGTAAATTGAAAAAACTTGATATGTC	

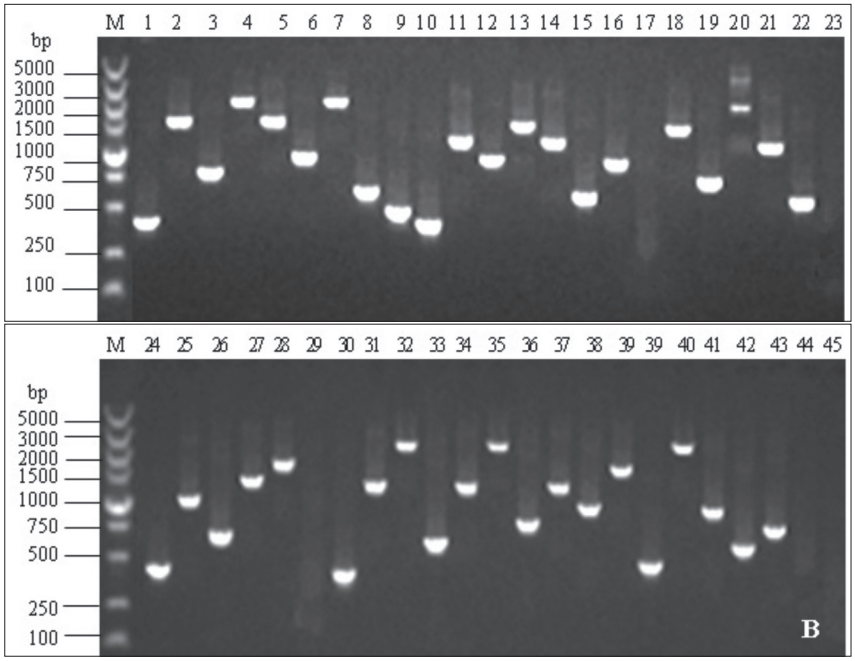


Fig 1. Identification of positive clones containing DNA fragments by PCR

M: DL5000 DNA marker. A. 1: pET28a(+) plasmid as control; 2~23: The clones selected randomly; B. 24: pET28a(+) plasmid as control; 25~45 The clones selected randomly

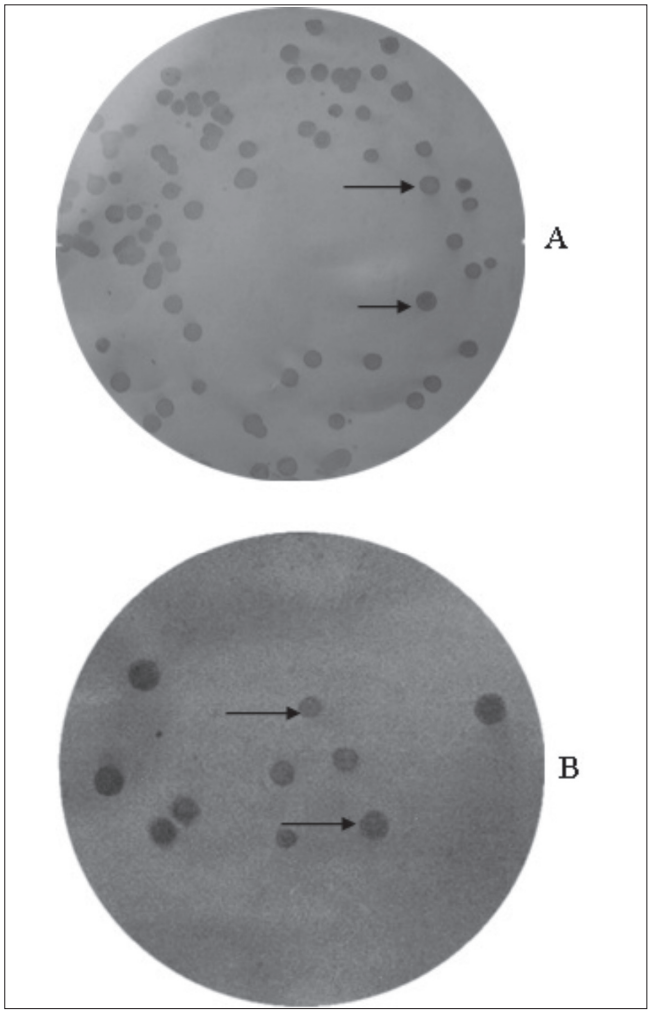


Fig 2. The screening of positive clones expressing DNA fragments of *M. ovipneumoniae* from whole genomic library. A: The preliminary screening of positive clones from whole genomic library; B: The re-screening of true positive clones from the positive clones

Screening of Positive Clones Containing Insert Sequences

Positive clones containing insert sequences were successfully screened by PCR from the genomic library *(Fig. 1 A, B)*. The analysis of insert sequences showed that they shared high identities (from 90% to 100%) with *M. ovipneumoniae* epidemic strains SC01, NM2010 and France14811. Among positive clones, a total of 10 clones were screened by immunoblotting assay, which could react with the *E. coli*-adsorbed *M. ovipneumoniae* positive serum *(Fig. 2A, B)*. Sequence analysis indicated that these 10 clones contained 5 different open reading frames of *M. ovipneumoniae* genome.

Sequence Analysis of Genes Encoding Immunogenic Proteins

The identities of the 5 immune-associated proteins were shown in *Table 2*. Sequence analysis showed that P56 protein contains PotE conserved domain that is involved in amino acid transport and P97-like protein contains a transmembrane region at the position of 21-43 amino acids. Based on our knowledge, this is the first time that IF-1 was identified from *M. ovipneumoniae*. IF-1 contains an S1-like RNA-binding domain, which is found in a wide variety of RNA-associated proteins. These domains described above play an important role in relevant *M. ovipneumoniae* biological processes.

Immunogenicity Analysis of M. ovipneumoniae Immune-Related Antigens

MAP, IF-1 and P97-like proteins were successfully expressed in *E. coli (Fig. 3)*, respectively. Western blot showed that all the three proteins have reactivity with *M. ovipneumoniae* positive serum *(Fig. 3A, B, C)*. The results of immunogenicity analysis showed that MAP could stimulate strong immune response, inducing an antibody titer of 1:32. In contrast,

Table 2. *Overview of the immunogenic proteins identified in M. ovipneumoniae*

***M. ovipneumoniae* Strain shz-1**					**Identity to Other Strains**		
Number	**Insert (bp)**	**Genome Llocation**	**Gene Product**	**Predicted Function**	**Strain SC01 Locus Tag (% Identity)**	**Strain NM2010 Locus Tag (% Identity)**	**Strain 14811 Locus Tag (% Identity)**
1	228	20098-20325	Translation initiation factor IF-1	Translation-related protein	MOSC01_RS0103275 (100%)	AG35_RS00360 (99%)	MOVI_1910 (97%)
2	630	19345-19974	Methionine aminopeptidase	Unknown	MOSC01_RS0103270 (97%)	AG35_RS00360 (99%)	MOVI_1910 (97%)
3	702	7677-8378	Hypothetical protein (P97-like protein)	Transmembrane protein	MOSC01_RS0103395 (93%)	AG35_RS03010 (96%)	MOVI_4210 (98%)
4	921	19813-20733	P56 membrane protein	Amino acid transporters-related protein	MOSC01_RS0103630 (99%)	AG35_RS03280 (95%)	MOVI_0270 (95%)
5	975	11471-12445	Hypothetical protein (P102-like protein)	Transmembrane protein	MOSC01_RS0103400 (100%)	AG35_RS03005 (90%)	MOVI_4200 (91%)

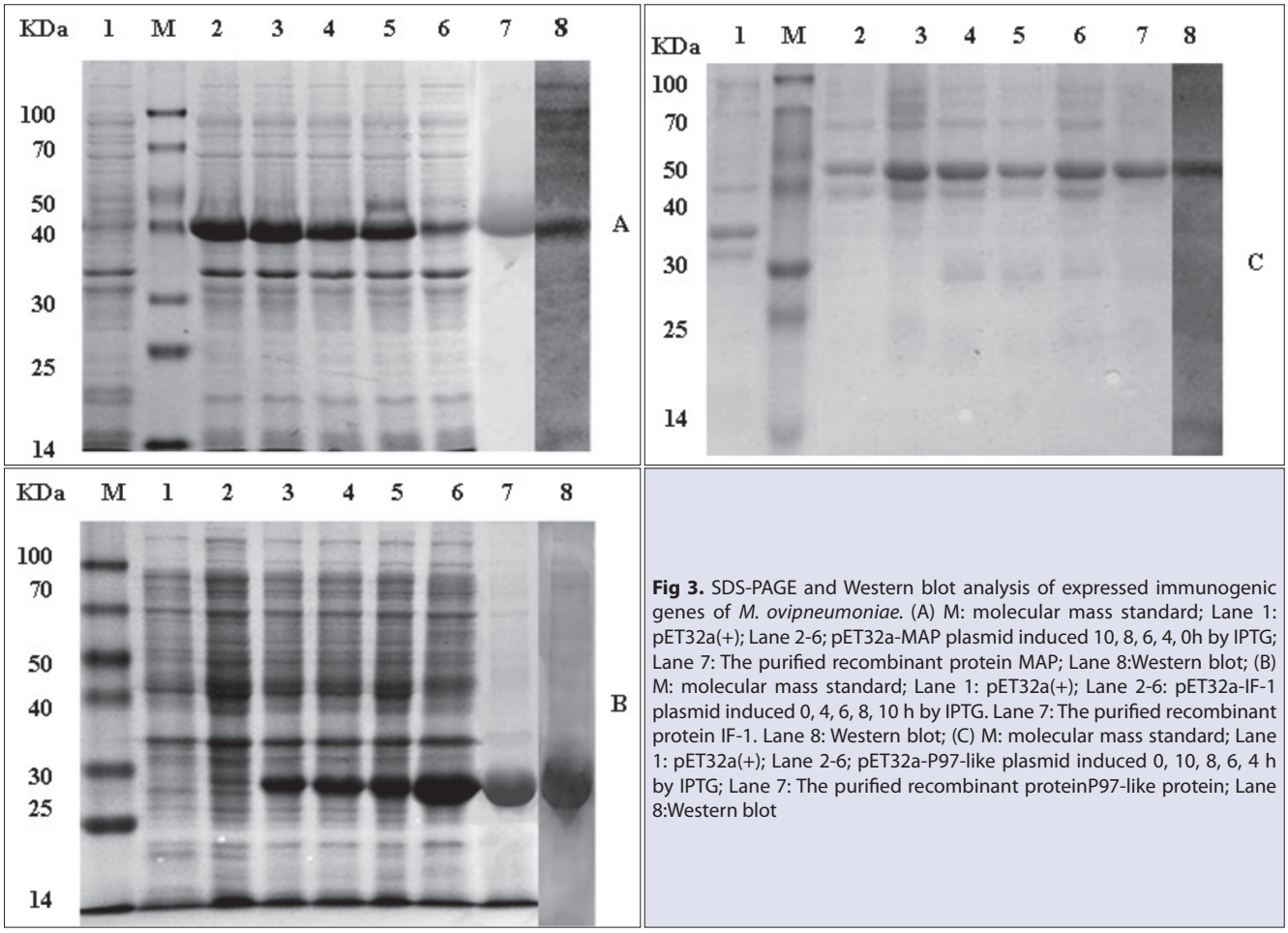


Fig 3. SDS-PAGE and Western blot analysis of expressed immunogenic genes of *M. ovipneumoniae*. (A) M: molecular mass standard; Lane 1: pET32a(+); Lane 2-6; pET32a-MAP plasmid induced 10, 8, 6, 4, 0h by IPTG; Lane 7: The purified recombinant protein MAP; Lane 8:Western blot; (B) M: molecular mass standard; Lane 1: pET32a(+); Lane 2-6: pET32a-IF-1 plasmid induced 0, 4, 6, 8, 10 h by IPTG. Lane 7: The purified recombinant protein IF-1. Lane 8: Western blot; (C) M: molecular mass standard; Lane 1: pET32a(+); Lane 2-6; pET32a-P97-like plasmid induced 0, 10, 8, 6, 4 h by IPTG; Lane 7: The purified recombinant proteinP97-like protein; Lane 8:Western blot

IF- 1 and P97-like protein produced relatively low antibody titer *(Fig. 4A, B, C)*.

DISCUSSION

Identification of immune-associated proteins may contribute to the development of diagnostic reagents and vaccines for infectious agents. At present, genomic expression library has been frequently used to screen and identify pathogens' immunogenic proteins. Ron et al.[20] employed *in vivo* induced antigen technology (IVIAT) and identified 13 proteins from the genomic expression library of *M. gallisepticum*, among which five hypothetical virulence factors (GapA, PlpA, Hlp3, VlhA 1.07 and VlhA 4.01) that have been previously described and eight new virulence factors (transport protein PotE, MGA_0241, 0654, translation protein L2, L23, ValS, chaperon GroEL and MGA_0042, a protein with unknown function). Kügler et al.[21] identified 6 immunogenic proteins from genomic library of *M. hyopneumoniae*. In the present study, we screened genomic

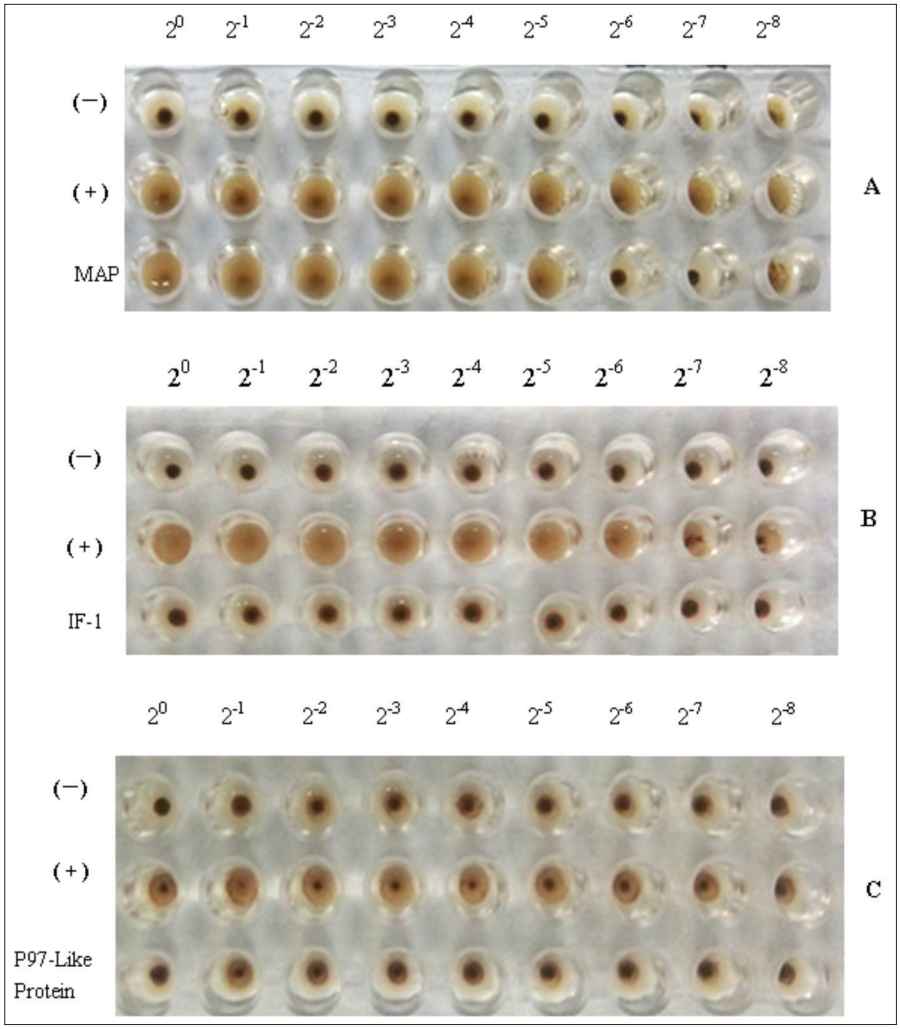


Fig 4. Detection of antibody titers in sera from lambs immunized by recombinant proteins of *M. ovipneumoniae*. (A) MAP protein; (B) IF-1 protein; (C) P97-like protein

expression library using *M. ovipneumoniae* positive serum and identified 5 proteins, including intracellular protein (Methionine Aminopeptidase), two hypothetical proteins (P97-like protein, P102-like protein), a membrane protein (P56) and the translation initiation factor IF-1. The sequence of these proteins shared 90-100% identities with MO strain SC01, NM2010 and France 14811.

Although the complete genome of the *M. ovipneumoniae* France 14811, SC01 and NM2010 strains have been sequenced [14,15], the genes that encode protective antigens remain unclear. Given that *M. ovipneumoniae* lacks cell wall structure, antigenic proteins are mainly in the cell membrane. Yang et al.[22] performed *in silico* analysis of MO genome and predicted virulence associated proteins including P146 adhesin like-protein, P97-like protein, adhesin, P76 and P113 protein [14]. However, these proteins have not been immunologically validated. Some studies have confirmed P97 protein is an essential virulence factor, which is involved in invasion process of *Mycoplasma hyopneumoniae*. P56 membrane protein, identified in this study, is a permease containing multiple transmembrane domains. The PotE domain of P56 is presumed to be involved in the transport and metabolism of polyamines, whereas polyamine is involved in gene expression, specific binding with proteins and cell permeability. The second protein identified in this study, MAP, is an important cytosolic enzyme, which is mainly involved in the N-terminal methionine cleavage and formation of the mature protein. It is worth noting that IF-1, which participates in protein translation [23], displays strong reactivity with *M. ovipneumoniae* positive serum but weak immunogenicity in animal experiment.

EF-Tu, HSP70, PDHA and PDHB have been reported to be immune-associated proteins in *M. ovipneumoniae* [24]; however, these proteins were not identified in this study. This is possibly due to the fact that *M. ovipneumoniae* uses UGA as the preference amino acid codon for tryptophan. In *E. coli*, UGA is a stop codon, which prevents gene expression in *E. coli* host during screening. Furthermore, some genes may be disrupted by the restriction endonuclease, which is responsible for the limitations of genomic expression library. Further studies (e.g., Immunization and challenges) are required to determine the protective antigens in lambs against *M. ovipneumoniae* infection.

In conclusion, the present study identified five immune-

associated proteins from *M. ovipneumoniae* genomic expression library. Among these proteins, MAP, a novel protein, showed strong immunogenicity in lambs, which displayed the potential value for the development of serological diagnostics and subunit vaccine against *M. ovipneumoniae* infection.

Acknowledgments

We thank the field staff who provided the technical assistance for this study. This work was supported by the International Science & Technology Cooperation Program of China (No.2014DFR31310), National Natural Science Foundation of China (No.31260601, 31460654), the Program for Youth innovation leader Science and Technology of XPCC (No. 2016BC001).

Conflict of Interests Statement

The authors declare that they have no conflict of interest.

REFERENCES

1. Alley MR, Ionas G, Clarke JK: Chronic non-progressive pneumonia of sheep in New Zealand - A review of the role of *Mycoplasma ovipneumoniae*. *N Z Vet J*, 47 (5): 155-160, 1999. DOI: 10.1080/00480169.1999.36135

2. Parham K, Churchward CP, McAuliffe L, Nicholas RA, Ayling RD: A high level of strain variation within the *Mycoplasma ovipneumoniae* population of the UK has implications for disease diagnosis and management. *Vet Microbiol*, 118 (1-2): 83-90, 2006. DOI: 10.1016/j.vetmic.2006.07.005

3. Gonçalves R, Mariano I, Núñez A, Branco S, Fairfoul G, Nicholas R: Atypical non-progressive pneumonia in goats. *Vet J*, 183 (2): 219-221, 2010. DOI: 10.1016/j.tvjl.2008.10.005

4. Rifatbegovic M, Maksimovic Z, Hulaj B: *Mycoplasma ovipneumoniae* associated with severe respiratory disease in goats. *Vet Rec*, 168 (21): 565, 2011. DOI: 10.1136/vr.d886

5. Niang M, Rosenbusch RF, DeBey MC, Niyo Y, Andrews JJ, Kaeberle ML: Field isolates of *Mycoplasma ovipneumoniae* exhibit distinct cytopathic effects in ovine tracheal organ cultures. *Zentralbl Veterinarmed A*, 45 (1): 29-40, 1998.

6. Ettorre C, Sacchini F, Scacchia M, Della Salda LD: Pneumonia of lambs in the Abruzzo region of Italy: Anatomopathological and histopathological studies and localisation of *Mycoplasma ovipneumoniae*. *Vet Ital*, 43(1): 149-155, 2007.

7. Rong G, Zhao JM, Hou GY, Zhou HL: Seroprevalence and molecular detection of *Mycoplasma ovipneumoniae* in goats in tropical China. *Trop Anim Health Prod*, 46 (8): 1491-1495, 2014. DOI: 10.1007/s11250-014-0645-y

8. Cheng C, Jun Q, Qingling M, Zhengxiang H, Yu M, Xuepeng C, Zibing C, Jinsheng Z, Zaichao Z, Kuojun C, Chuangfu C: Serological and molecular survey of sheep infected with *Mycoplasma ovipneumoniae* in Xinjiang, China. *Trop Anim Health Prod*, 47 (8): 1641-1647, 2015. DOI: 10.1007/s11250-015-0908-2

9. Jones GE, Gilmour JS, Rae AG: The effect of *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* on specific pathogen-free lambs. *J Comp Pathol*, 92 (2): 261-266, 1982. DOI: 10.1016/0021-9975(82)90084-6

10. Foreyt WJ, Snipes KP, Kasten RW: Fatal pneumonia following inoculation of healthy bighorn sheep with *Pasteurella haemolytica* from healthy domestic sheep. *J Wildl Dis*, 30 (2): 137-145, 1994. DOI: 10.7589/0090-3558-30.2.137

11. Weiser GC, DeLong WJ, Paz JL, Shafii B, Price WJ, Ward AC: Characterization of *Pasteurella multocida* associated with pneumonia in bighorn sheep. *J Wildl Dis*, 39 (3): 536-544, 2003. DOI: 10.7589/0090-3558-39.3.536

12. Dassanayake RP, Shanthalingam S, Herndon CN, Subramaniam R, Lawrence PK, Bavananthasivam J, Cassirer EF, Haldorson GJ, Foreyt WJ, Rurangirwa FR, Knowles DP, Besser TE, Srikumaran S: *Mycoplasma ovipneumoniae* can predispose bighorn sheep to fatal *Mannheimia haemolytica* pneumonia. *Vet Microbiol*, 145 (3-4): 354-359, 2010. DOI: 10.1016/j.vetmic.2010.04.011

13. Besser TE, Frances Cassirer E, Highland MA, Wolff P, Justice-Allen A, Mansfield K, Davis MA, Foreyt W: Bighorn sheep pneumonia: Sorting out the cause of a polymicrobial disease. *Prev Vet Med*, 108 (2-3): 85-93, 2013. DOI: 10.1016/j.prevetmed.2012.11.018

14. Yang F, Tang C, Wang Y, Zhang H, Yue H: Genome sequence of *Mycoplasma ovipneumoniae* strain SC01. *J Bacteriol*, 193 (18): 5018, 2011. DOI: 10.1128/JB.05363-11

15. Wang XH, Huang HB, Cheng C, Wang RC, Zheng JQ, Hao YQ, Zhang WG: Complete genome sequence of *Mycoplasma ovipneumoniae* strain NM2010, which was isolated from a sheep in China. *J Integr Agric*, 13 (11): 2562-2563, 2014. DOI: 10.1016/S2095-3119(14)60825-X

16. March JB, Jepson CD, Clark JR, Totsika M, Calcutt MJ: Phage library screening for the rapid identification and *in vivo* testing of candidate genes for a DNA vaccine against *Mycoplasma mycoides* subsp. *mycoides* small colony biotype. *Infect Immun*, 74 (1): 167-174, 2006. DOI: 10.1128/IAI.74.1.167-174.2006

17. Hu Y, Huang J, Jiao XA: Screening of genes expressed *in vivo* during interaction between chicken and *Campylobacter jejuni*. *J Microbiol Biotechnol*, 24 (2): 217-224, 2014. DOI: 10.4014/jmb.1308.08092

18. Guimaraes AMS, Santos AP, Timenetsky J, Bower LP, Strait E, Messick JB: Identification of *Mycoplasma suis* antigens and development of a multiplex microbead immunoassay. *J Vet Diagn Invest*, 26 (2): 203-212, 2014. DOI: 10.1177/1040638713520542

19. Studier FW, Moffatt BA: Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol*, 189 (1): 113-130, 1986. DOI: 10.1016/0022-2836(86)90385-2

20. Ron M, Gorelick-Ashkenazi A, Levisohn S, Nir-Paz R, Geary SJ, Tulman E, Lysnyansky I, Yogev D: *Mycoplasma gallisepticum in vivo* induced antigens expressed during infection in chickens. *Vet Microbiol*, 175 (2-4): 265-274, 2015. DOI: 10.1016/j.vetmic.2014.12.007

21. Kügler J, Nieswandt S, Gerlach GF, Meens J, Schirrmann T, Hust M: Identification of immunogenic polypeptides from a *Mycoplasma hyopneumoniae* genome library by phage display. *Appl Microbiol Biotechnol*, 80 (3): 447-458, 2008. DOI: 10.1007/s00253-008-1576-1

22. Minion FC, Adams C, Hsu T: R1 region of P97 mediates adherence of *Mycoplasma hyopneumoniae* to swine cilia. *Infect Immun*, 68 (5): 3056-3060, 2000. DOI: 10.1128/iai.68.5.3056-3060.2000

23. Celano B, Pawlik RT, Gualerzi CO: Interaction of *Escherichia coli* translation-initiation factor IF-1 with ribosomes. *Eur J Biochem*, 178 (2): 351-355, 1988. DOI: 10.1111/j.1432-1033.1988.tb14457.x

24. Li M, Ma CJ, Liu XM, Zhao D, Xu QC, Wang YJ: Molecular cloning of HSP70 in *Mycoplasma ovipneumoniae* and comparison with that of other mycoplasmas. *Genet Mol Res*, 10 (2): 834-848, 2011. DOI: 10.4238/vol10-2gmr1193

Kafkas Univ Vet Fak Derg
26 (2): 209-215, 2020
DOI: 10.9775/kvfd.2019.22766

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Interconnected Multiple Gastroesophageal Fish Hook Retrievals by Endoscopic or Surgical Interventions In Dogs: 13 Cases (2010-2017) [1]

Cenk YARDIMCI [1,a] Kamil Serdar İNAL [1,b] Taylan ÖNYAY [1,c] Ahmet ÖZAK [1,d]

[1] *The study was presented as a poster in the II[nd] International and XVI[th] National Veterinary Surgery Congress of Turkey, 20-23[th] September 2018, Bafra, North Cyprus*

[1] Ondokuz Mayıs University Faculty of Veterinary Medicine, Department of Surgery, TR-55200 Atakum, Samsun - TURKEY
[a] ORCID: 0000-0001-6126-3603; [b] ORCID:0000-0003-0690-5743; [c] ORCID: 0000-0001-5638-7257; [d] ORCID:0000-0002-4359-0691

Article ID: KVFD-2019-22766 Received: 30.05.2019 Accepted: 30.09.2019 Published Online: 01.10.2019

How to Cite This Article

Yardimci C, İnal KS, Önyay T, Özak A: Interconnected multiple gastroesophageal fish hook retrievals by endoscopic or surgical interventions in dogs: 13 cases (2010-2017). *Kafkas Univ Vet Fak Derg,* 26 (2): 209-215, 2020. DOI: 10.9775/kvfd.2019.22766

Abstract

The aim of this study was to evaluate endoscopic and/or surgical removal of interconnected, multiple fish hooks that lodge in gastroesophageal region in dogs. Medical records were obtained and clinical, radiographic and endoscopic evaluations were performed for 13 dogs with gastroesophageal multiple fish hooks. Endoscopic, surgical and endoscopy assisted surgical removal was performed depending on penetration degree of the hooks. Distribution of the hooks were 9 (13.2%) in the cranial esophagus, 40 (58.8%) in the cervical esophagus, 17 (25%) in the thoracic esophagus and 2 (3%) in the stomach. Retrieval of the hooks was performed endoscopically in 11 cases, surgically in one case and combined in one case. There were no complicated mucosal erosions or lacerations associated with removal of the hooks. It is very important to remove fish hooks as soon as possible to reduce complication rates and the dog owners should be warned to beware of fishing areas.

Keywords: *Dog, Endoscopy, Esophagus, Fish hooks, Foreign body*

Köpeklerde Birbirine Bağlı Gastroözefageal Çoklu Balık Kancalarının Endoskopik ve Cerrahi Girişimlerle Uzaklaştırılması: 13 Olgu (2010-2017)

Öz

Bu çalışmanın amacı, köpeklerde gastroözefageal bölgeye takılan birbirlerine bağlı çoklu balık kancalarının endoskopik ve cerrahi girişimlerle uzaklaştırılmasının değerlendirilmesidir. Hastaların kayıtları alındıktan sonra gastroözefageal çoklu balık kancası olan 13 köpeğin radyografik ve endoskopik değerlendirilmeleri yapıldı. Kancaların penetrasyon derecesine bağlı olarak endoskopik, cerrahi ve endoskopi yardımlı cerrahi uzaklaştırma uygulandı. Kancaların dağılımları; kranial özofagusta 9 (%13.2), servikal özefagusta 40 (%58.8), torakal özofagusta 17 (%25) ve midede 2 (%3) idi. Kancaların uzaklaştırılması 11 olguda cerrahi, bir olguda endoskopik olarak gerçekleştirildi ve bir olguda kombine edildi. Kancaların uzaklaştırılmasına bağlı olarak komplike mukozal erozyon veya laserasyon gözlenmedi. Komplikasyon oranını azaltmak için balık kancalarını mümkün olan en kısa sürede çıkarmak oldukça önemlidir ve köpek sahipleri balık avlama alanlarında dikkatli olmaları için uyarılmalıdır.

Anahtar sözcükler: *Balık kancası, Endoskopi, Köpek, Özefagus, Yabancı cisim*

INTRODUCTION

Household pets are prone to foreign body ingestion and this occurrence is relatively common in dogs [1-3]. Bones are the most commonly encountered esophageal foreign bodies (EFB)s, although balls, toys, fish hooks and wood sticks were also reported [3-5]. Some dogs may remain asymptomatic but most show acute clinical symptoms that include gagging, hypersalivation, dysphagia and discomfort on palpation [3]. When the symptoms are obvious, EFB is considered as an emergency case in veterinary medicine [3,5-7].

Clinical symptoms and complications of ingested fish hooks vary due to the number, size and localization of fish hooks, time elapsed since ingestion, depth of penetration and the presence and magnitude of perforation [8,9]. The most likely places of ingested fish hooks to lodge are

İletişim (Correspondence)
+90 362 3121919 (1228)
cyardimci@omu.edu.tr

the base of the heart, thoracic inlet and esophageal hiatus because of natural anatomical strictures [5,8]. While fish hooks can easily be detected in radiographic examination, endoscopy is still the golden diagnostic method of choice because it allows visualization of the hook and proper detection of mucosal damage [3]. Esophagitis, aspiration pneumonia, esophageal perforation, and esophageal stricture formations may occur due to damage from hooks [2,4,10-12].

Fishhook and bone more likely cause perforation in all EFBs. Endoscopic retrieval is preferred over surgery but it is not always possible because the hooks may be lodged in the mucosa and retrieval may cause tear damage. Barbed shape of the hooks disallows simple removal in most cases as they are designed to lodge in place. If endoscopic retrieval fails, and/or seemed to be too risky, surgical intervention must be considered [5]. The fish hook should be removed as soon as possible as movement due to peristaltic action may cause or worsen mucosal damage [2,4,10].

The purpose of this study was to demonstrate the clinical experience of endoscopic, endoscopy assisted surgical or surgical removal of multiple fish hooks that were attached to one another in the gastroesophageal area. Additionally, it is unique because this is the first paper to report ingestion of multiple hooks attached with a line to each other.

MATERIAL and METHODS

Animals

The study material was composed of 13 dogs that were brought to Animal Hospital of Ondokuz Mayıs University with suspicion of foreign body ingestion. They were confirmed to have interconnected multiple fish hooks in different sizes between their esophagus and pylorus. Signalment of the patients, time between ingestion and clinical intervention and methods used for removal are presented at *Table 1*.

After patient histories were noted, complete physical evaluations and oral cavity examinations were performed for each patient. The localization of fish hooks were noted in the general areas of the cranial esophagus (caudal pharynx), cervical esophagus (between cranial esophagus and thoracic inlet), thoracic esophagus (between thoracic inlet and diaphragm), caudal esophagus (between diaphragm and cardia) and stomach.

Grading of the Patients

Savary-Miller classification was used to grade esophageal lesions related to fish hooks [13]. According to this, single erosions were classified as grade I, confluent erosions were

Table 1.*Case details of the dogs with ingested fish hooks*

Case No	Signalment	Number of the Hooks	Hook Size	Localization of the Hooks	Duration of Time From Ingestion to Removal Attempt (Approximately)	Technique/Duration of the Procedure
1	Doberman Pinscher, 4-m-old, M, 32 kg	2 hooks	No:4 x 2	Cranial esophagus x 2	4 h	Endoscopic (10 min)
2	Mix breed, 18-m-old, M, 23 kg	4 hooks	No:4 x 4	Cervical esophagus x 4	8 h	Endoscopic (17 min)
3	Labrador Retriever, 3-y-old, M, 30 kg	10 hooks	No:4 x 10	Cervical esophagus x 2, Thoracic esophagus x 8	2 h	Endoscopic (75 min) + surgical (65 min)
4	Golden Retriever, 8-y-old, M, 33 kg	7 hooks	No:7 x 1 No:4 x 5 No:3 x 1	Cranial esophagus x 2, Cervical esophagus x 5	24 h	Endoscopic (26 min)
5	Mix breed, 9-m-old, F, 18 kg	5 hooks	No:4 x 5	Cervical esophagus x 5	18 h	Endoscopic (34 min)
6	Terrier, 15-m-old, M, 8 kg	3 hooks	No:3 x 3	Thoracic esophagus x 3	4 h	Endoscopic (20 min)
7	Labrador Retriever, 10-m-old, F, 22 kg	5 hooks	No:4 x 3 No:3 x 2	Cervical esophagus x 5	Unknown	Endoscopic (36 min)
8	Mix breed, 8-m-old, M, 14 kg	6 hooks	No:4 x 5 No:3 x 1	Cranial esophagus x 2 Cervical esophagus x 4	12 h	Endoscopic (19 min)
9	Golden Retriever, 14-m-old, M, 26 kg	8 hooks	No:7 x 8	Cervical esophagus x 8	24 h	Endoscopic (40 min)
10	Mix breed, 1-y-old, F, 15 kg	7 hooks	No:4 x 4 No:3 x 1 No:7 x 2	Cervical esophagus x 5 Thoracic esophagus x 2	2 h	Endoscopic (30 min)
11	Pointer, 18-m-old, F, 15 kg	2 hooks	No:3 x 2	Stomach x 2	Unknown	Surgical (40 min)
12	Mix breed, 2-y-old, M, 18 kg	6 hooks	No:3 x 6	Cervical esophagus x 2 Thoracic esophagus x 4	6 h	Endoscopic (27 min)
13	Mix breed, 15-m-old, M, 20 kg	3 hooks	No:4 x 3	Cranial esophagus x 3	Unknown	Endoscopic (10 min)

graded as II, while circular confluent erosions were graded as III and finally ulcerations, stenosis or perforations were graded as IV. Patients were evaluated according to their esophageal lesions as mild esophagitis (Grade I & II) or moderate esophagitis (Grade III & IV).

Anaesthesia

Initial endoscopic examination was performed under propofol anesthesia and soft tissue damage was visualized. If the fish hooks were not lodged in the soft tissue, endoscopic retrieval procedure was completed with propofol anesthesia. For fish hooks determined to be embedded in the soft tissue, the anesthesia was maintained with isoflurane (2% Isoflurane, Adeka®) and endoscopic retrieval procedure would then become a gastrotomy case. Simple endoscopic retrieval of the hooks was done under propofol anaesthesia (Propofol 1% Fresenius®) if the fish hooks and tangled lines could be removed easily. More complicated cases were intubated and maintained with isoflurane anesthesia (Isoflurane 2%, Adeka®).

Removal Process

Fish hooks which are not imbedded in the mucosa, were retrieved by endoscopy but the procedure was stopped immediately if pulling the entangled line and fish hooks would cause injury to the mucosa. If the fish hooks were lodged in, or pulling them would cause injury to the mucosa, surgery was performed. Endoscopic assistance was performed in some cases to reduce exposure of the surgery site.

Fish hooks were removed using three different methods; endoscopic, endoscopy assisted surgical, or surgical retrieval. If the hooks were retrieved solely by endoscopy, the procedure was classified as endoscopic retrieval. If the fish hook was removed surgically, it was classified as surgical retrieval. If the hooks were advanced to the caudal esophagus or cardia using endoscopy then removed via surgical intervention, this is classified as endoscopy assisted surgical retrieval.

In all patients, before any retrieval attempt, fishing line attachments were cut and hooks were separated in order to avoid complications during removal procedure. Endoscopic retrieval was performed by two techniques. In the first technique, free fish hooks were retrieved using flexible grasping forceps thorough the working channel of the endoscope. In the second technique, lodged fish hooks were retrieved with rigid grasping forceps without using the working channel of the endoscope. Retrograde endo-scopic retrieval was performed with gastrotomy if the hooks could not be retrieved with endoscopy due to them being lodged in or would cause severe soft tissue damage upon retrieval.

Postoperative Care

After fish hooks retrieval, food is restricted for 24 h, after 12 h oral fluids has been given. Amoxicillin Clavulanic acid (Synulox, 12.5 mg oral tablet, Zoetis®) was administered for 5-7 days. In gastrotomy cases, meloxicam was also given for 3 days postoperatively at a dose of 0.1 mg/kg/day (Maxicam, 5 mg/mL, inj, Sanovel®, Turkey). The patients that endoscopic retrieval was performed on were discharged following recovery from the anaesthesia while the patients that required gastrotomy were hospitalized for 2 days.

Long term evaluation of patients was performed by clinical examinations, information from referring veterinarian or phone surveys with patient owners. Patient owners were asked if there were any signs of dysphagia, coughing, regurgitation or vomiting, especially during eating or drinking, and if any medical interventions were made 6 months following the removal of fish hooks.

The postoperative periods of the patients were graded according to their ability to consume food. As such, the patients that were able to consume any food without any difficulty was graded as very good. Patients that had some difficulty eating solid food such as kibbles with gag reflex but without vomiting were graded as good. The patients that could only consume soft food and liquids and gagged and vomited when eating solid food was graded as fair. Those having difficulty when eating even soft food and liquids are graded as poor.

RESULTS

The first clinical examination of patients revealed the symptoms of hypersalivation, and dampness of the front limbs due to this, unwillingness to eat or drink, reluctance to move the head with intermittent retching and gagging. Twelve of the 13 dogs in this study were large breed dogs and the male/female ratio was 9/4. The average age and body weights of the patients were 21.5 months and 21 kg, respectively. According to the international fish hook sizing chart, 41 of the hooks removed were size 4, 16 were size 3 and 11 were size 7 out of a total 68 fish hooks. The localization of fish hooks in the GI tract were as follows; 9 (13.2%) in the cranial, 40 (58.8%) in the cervical, 17 (25%) in the thoracic esophagus and 2 (3%) in the stomach. Aside from 3 cases (case no 7, 11 and 13) which the owners didn't know the exact time of occurrence, the time from ingestion to removal time was between 2-24 h (mean 10 h). The hooks were removed with endoscopy in 11 cases, endoscopy assisted surgery in one case and surgery in one case.

According to the patient histories, each owner stated that the probable time of ingestion was noticed but was deemed unimportant at the time because the ingested object was thought to be a piece of bread or fish. However since the incidents happened near common fishing spots, owners suspected something was wrong at the occurrence of the first clinical symptoms and brought their dogs immediately after.

Endoscopic Technique

This procedure was successfully performed in 11 of 12 (91.6%) patients to remove all fish hooks. In the remaining case (case no:3) five of the hooks were retrieved endoscopically and the other 5 were removed with endoscopy assisted gastrotomy. Endoscopic retrieval was not attempted in gastric cases due to possible complications. During endoscopic removal, barbs of the free hooks were held with the grasping forceps to avoid damaging the mucosal surface. The greatest challenge of endoscopic retrieval of attached hooks was their synchronized response to manipulations. In other words, the maneuvers made to remove one hook might have mobilized the others that are attached and that could have potentially damaged the mucosa or even lodged other hooks into it. To avoid this complication, lines attaching the hooks to each other were cut first. Freed single hooks were removed with a flexible grasping forceps *(Fig. 1)*. However, this could not be done in lines that were tangled to each other in multiple locations *(Fig. 2a,b,c)*. In these cases, multiple hooks were tried to be removed at the same time *(Fig. 2d)*. Superficial mucosal damage was either already present due to the fish hook ingestion *(Fig. 3)* or caused by the endoscopic retrieval *(Fig. 4)* due to barbs of the hooks in each case. However this superficial scarring was considered only as a mild disruption to the esophagus that did not require any kind of special treatment. The insertion of the endoscope from the mouth initiated the endoscopy duration and removal of the last hook marked the end. This duration changed depending on the number, size, localization of hooks, mucosal penetration level and the amount of entanglement. Endoscopy durations varied between 10 to 75 min with an average of 29 min.

Endoscopy Assisted Surgery

This was performed on 1 patient (case no:3) that swallowed 10 fish hooks *(Fig. 5a)* in total and endoscopic retrieval was only suitable for 5 of them. For removal of the remaining hooks that were lodged in diaphragmatic hiatus *(Fig. 5b)*,

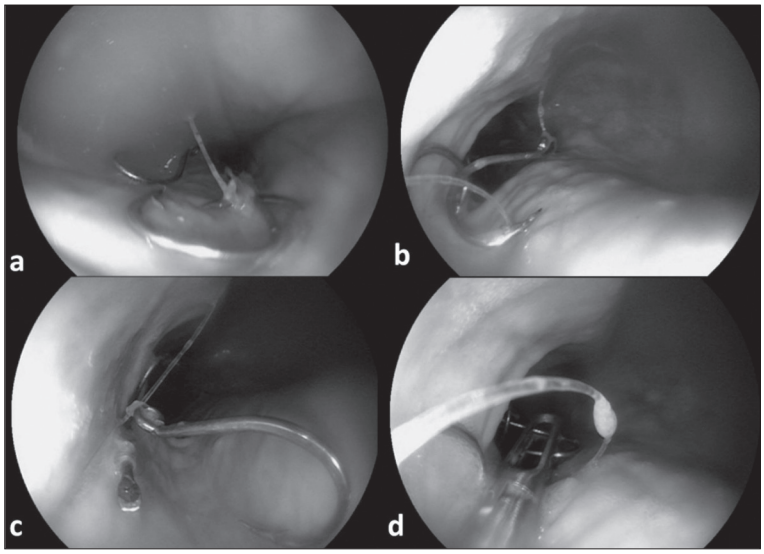
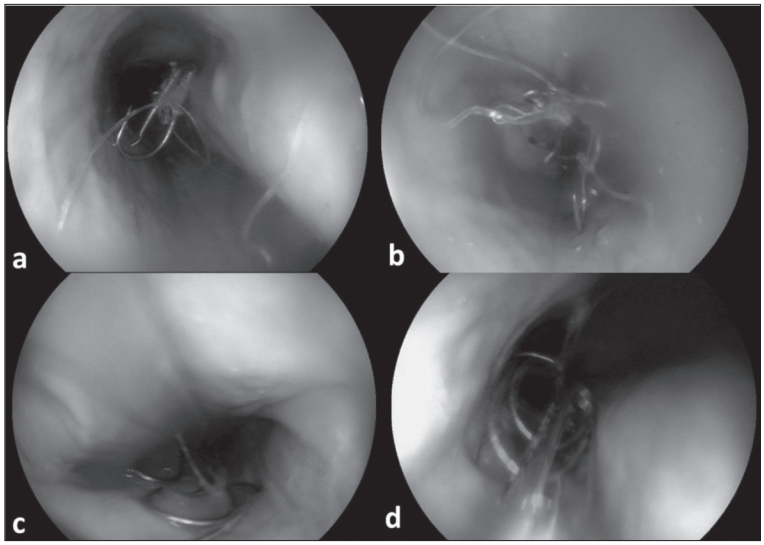


Fig 1. Free fish hooks were removed by taking care of the barbs for damaging the esophageal mucosa. First, lines were cut (a-c) and then hooks were grasped with forceps by securing the barb (d)

Fig 2. Some of the hooks were tangled (a-c) and could not be moved separately. In such cases multiple hooks were grasped by avoidance of the sharp edge of the barbs (d)



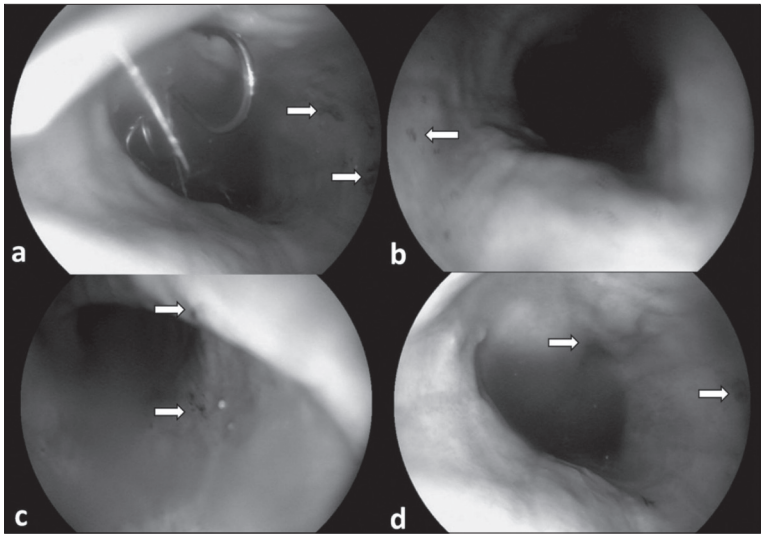
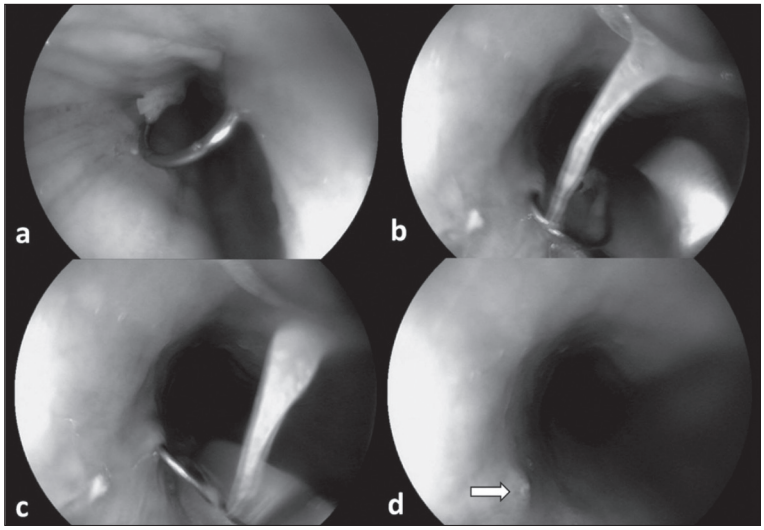


Fig 3. Duringendoscopic examination Savary-Miller Grade-I superficial erosions *(arrows)* of the esophageal mucosa owing to the free hooks were observed

Fig 4. Some of the lodged hooks were removed by gentle maneuvers (a-c). After removal of the barb negligible mucosal damage *(arrow)* was seen (d)



retrograde esophagoscopy was performed following gastrotomy. The endoscope was passed through the cardia to the esophagus. The hooks lodged into the mucosa were removed with a rigid grasping forceps using slight force to the opposite side of the barbs to prevent serious damage to the mucosa. All were removed without any complication. The patient started consuming soft food at the 24th h and no long-term complications were observed.

Surgical Technique

This technique was performed in one case (case no: 11) with 2 fish hooks in the stomach. The stomach was exposed via a standard laparotomy and gastrotomy. One of the hooks was found embedded in the gastric muscle layer but the one attached to it was free. The free hook was seen to lose its sharp point, probably due to corrosion inside the stomach. The fish hook that pierced the gastric muscles was not pulled back but pushed forward in a circular fashion as to push its reverse barb out of the mucosa to cut it, then pulled back to the opposite side to remove it without tearing the gastric muscles or mucosa. Then, the stomach and abdomen was routinely closed. The patient's condition was checked with regular phone surveys and its condition was found to be very well in the long term.

When long term results were evaluated, it was observed that all patients were very good. And no complications related to esophageal lesions were seen in any of the dogs.

DISCUSSION

The most common gastric and esophageal foreign bodies reported in dogs are bones, cartilage, chew treats and toys; the incidence of fish hooks are relatively low compared to the aforementioned foreign bodies [3,8]. This is probably due to the fact that fish hook usage is limited to lakes and coastal areas. No published data of esophageal multiple fish hook injuries of dogs have been reported until now [1,3,5,6,8]. In addition, the number of the gastric multiple fish hooks cases were reported to be only three [5]. This study evaluates only multiple fish hooks connected to each other with lines and whilst the case number may seem low, this retrospective

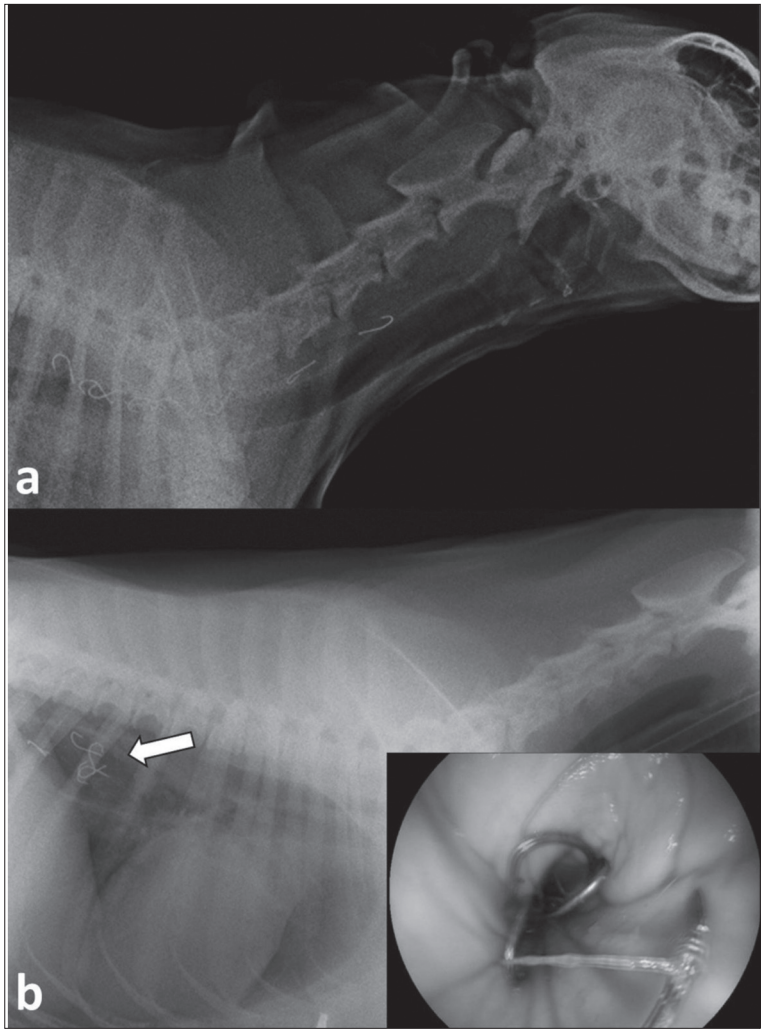


Fig 5. Lateral radiograph of a dog after ingestion of ten no: 4 fish hooks at the initial examination (a). Five of the hooks could remove endoscopically but the remaining five were tangled and stucked in the diaphragmatic hiatus (b). Following gastrotomy, retrograde esophagoscopy was performed for removal of the remaining hooks

study has the largest number of cases with interconnected multiple fish hook ingestion to date.

We could not find any etiological data concerning fish hook ingestion in dogs in the literature [5], but it is extremely improbable for a dog to find and ingest a fish hook without the human factor. Coastal fishing is very popular in the region where this study was done and is active all year round. According to the patient histories, most foreign body ingestions occurred due to fishing bait hiding hooks that were left behind by heedless fishermen. The most common etiological reason was the ingestion of multiple combination string hooks embedded in pieces of round bread pellet left unattended by fishermen, which can easily be swallowed by the dog because of its soft texture. Thus, it can be said that humans are responsible rather than animals in fish hook ingestion related injuries. Informing fishermen and dog owners about this subject should decrease the incidence of fish hook ingestion related injuries.

The initial lesions caused by the embedded fish hook is generally milder and more superficial than the ones caused during endoscopic removal. The combination of string's attachment and entanglement levels is as important as the number of the hooks and their penetration levels to the mucosa. As such, any manipulation in order to remove one hook attached to others may cause them to penetrate the mucosa because they are also pulled away. To avoid this complication, cutting the combination fishing line initially is of utmost importance. Unfortunately it is not always possible with the use of endoscopy when the line is badly entangled. In such cases we think it would be best to move on to surgical removal without wasting any more time.

Complications occurring due to EFBs are generally classified as obstructions, local ischemias and lacerations. The main reported complications related to that are esophagitis esophageal lacerations, aspiration pneumonia, esophageal stricture formation and rarely pneumothorax, pneumomediastinum, pleural effusion, pyothorax, hemothorax, pneumonia, bronchoesophageal fistula, aortaesophageal fistula, cardiopulmonary arrest and death [2,4,10,11,14-17]. Previous study suggests that the perforation only becomes attached to the fish hook and bone within all EFBs [18].

Due to barbed structure of the fish hooks, mucosal injuries

are very compatible with lacerations and perforations from obstruction or ischemia. According to the authors, the reason for only superficial mucosal erosions in present study was evaluated as an intervention made shortly after the swallowing of the hooks. So, it should not be forgotten that early diagnosis and intervention is the most important step in preventing possible complications.

In a retrospective study including fish hook ingestions of 75 dogs and 3 cats in a duration of 16 years, multiple fish hooks were only seen in 4 dogs [5]. In the same study, the fish hook localizations were as follows; 38 in the esophagus (all 38 had ingested only 1 fish hook), 39 in the stomach (36 had a single fish hook, 2 had 2 fishhooks each, and 1 had 5 fish hooks), and 1 fishhook in the distal portion of the esophagus with a second fish hook in the stomach. According to this study almost 50% of the fishhooks were found in the stomach, which does not correlate with our study when the location distribution is considered. The reason for this situation should be related to the local fishing habits in which a bait is used composed of multiple combination string hooks embedded in a piece of a bread pellet. After contacting with saliva, this pellet crumbles and the hooks release which set the stage for penetration to the esophageal mucosa. It was concluded that the fact that the number of fish hooks detected in the similar studies were much higher than those in our study was concealed in the baits that could be broken down by enzymatic digestion like fish.

There was no significant correlation between the number of hooks and their location between retrieval time but generally fish hooks that were imbedded and with entangled strings took longer to remove. The prognosis of ingested fish hooks may be better than other foreign body ingestions if treated expertly because the mucosal damage cannot be greater then the diameter of the needle in uncomplicated cases. That being said, the hooks should not be retrieved on the opposite direction of their barbs, if they are pulled like that, serious mucosal damage is unavoidable. If the fish hook pierced the mucosa or deeper into the muscle, maneuvering to remove the hook with endoscopy mostly end in failure or severe lacerations. While attempting to remove deeply lodged fish hooks, pushing the hook forward instead of pulling it back may cause less damage to the mucosa because of their shape. Using this maneuver for removal of the penetrated hooks did not complicate or cause any significant mucosal damage in any case of this study.

In conclusion, decisive and early treatment can decrease the morbidity of multiple hook ingestions. Endoscopic retrieval of fish hooks located in the esophagus is preferred because it has fewer complications than surgery. However, the main reason should not be discounted, with proper education of the fishermen and pet owners about this subject, these incidents can be avoided entirely.

REFERENCES

1. Rousseau A, Prittie J, Broussard JD, Fox PR, Hoskinson J: Incidence and characterization of esophagitis following esophageal foreign body removal in dogs: 60 cases (1999-2003). *J Vet Emerg Crit Care,* 17 (2): 159-163, 2007. DOI: 10.1111/j.1476-4431.2007.00227.x

2. Spielman B, Shaker E, Garvey M: Esophageal foreign body in dogs: A retrospective study of 23 cases. *J Am Anim Hosp Assoc,* 28 (6): 570-574, 1992.

3. Thompson HC, Cortes Y, Gannon K, Bailey D, Freer S: Esophageal foreign bodies in dogs: 34 cases (2004-2009). *J Vet Emerg Crit Care,* 22 (2): 253-261, 2012. DOI: 10.1111/j.1476-4431.2011.00700.x

4. Luthi C, Neiger R: Esophageal foreign bodies in dogs: 51 cases (1992-1997). *Eur J Comp Gastroenterol,* 3, 7-11, 1998.

5. Michels G, Jones B, Huss B, Wagner-Mann C: Endoscopic and surgical retrieval of fishhooks from the stomach and esophagus in dogs and cats: 75 cases (1977-1993). *J Am Vet Med Assoc,* 207 (9): 1194-1197, 1995.

6. Sale CSH, Williams JM: Results of transthoracic esophagotomy retrieval of esophageal foreign body obstructions in dogs: 14 cases (2000-2004). *J Am Anim Hosp Assoc,* 42 (6): 450-456, 2006. DOI: 10.5326/0420450

7. Teh H, Winters L, James F, Irwin P, Beck C, Mansfield C: Medical management of esophageal perforation secondary to esophageal foreign bodies in 5 dogs. *J Vet Emerg Crit Care,* 28 (5): 464-468, 2018. DOI: 10.1111/vec.12757

8. Gianella P, Pfammatter NS, Burgener IA: Oesophageal and gastric endoscopic foreign body removal: Complications and follow-up of 102 dogs. *J Small Anim Pract,* 50 (12): 649-654, 2009. DOI: 10.1111/j.1748-5827.2009.00845.x

9. King J: Esophageal foreign body and aortic perforation in a dog. *Vet Med,* 96 (11): 828-829, 2001.

10. Cohn LA, Stoll MR, Branson KR, Roudabush AD, Kerl ME, Langdon PF, Johannes CM: Fatal hemothorax following management of an esophageal foreign body. *J Am Anim Hosp Assoc,* 39 (3): 251-256, 2003. DOI: 10.5326/0390251

11. Doran IP, Wright CA, Moore AH: Acute oropharyngeal and esophageal stick injury in forty-one dogs. *Vet Surg,* 37 (8): 781-785, 2008. DOI: 10.1111/j.1532-950X.2008.00448.x

12. Binvel M, Poujol L, Peyron C, Dunie-Merigot A, Bernardin F: Endoscopic and surgical removal of oesophageal and gastric fishhook foreign bodies in 33 animals. *J Small Anim Pract,* 59 (1): 45-49, 2018. DOI: 10.1111/jsap.12794

13. Savary M, Miller G: The Esophagus: Handbook and Atlas of Endoscopy. 248, Gassmann, Switzerland, 1978.

14. Tams TR: Endoscopy and laparoscopy in veterinary gastroenterology. **In,** Tams TR (Ed): Handbook of Small Animal Gastroenterology. 2nd ed., 97-117, Elsevier, Philadelphia, 2003.

15. Dunlap AE, Risselada M: Caudal mediastinal fish hook foreign body with pulmonary artery penetration in two dogs. *J Am Anim Hosp Assoc,* 55 (1): e551-01, 2019. DOI: 10.5326/jaaha-ms-6787

16. Boyd C, Claus M, Hosgood G, Smart L: Survival of two dogs with pyothorax secondary to perforating oesophageal foreign body. *Aust Vet J,* 95 (1-2): 41-45, 2017. DOI: 10.1111/avj.12548

17. Burton AG, Talbot CT, Kent MS: Risk factors for death in dogs treated for esophageal foreign body obstruction: A retrospective cohort study of 222 cases (1998-2017). *J Vet Intern Med,* 31 (6): 1686-1690, 2017. DOI: 10.1111/jvim.14849

18. Sterman AA, Mankin KMT, Ham KM, Cook AK: Likelihood and outcome of esophageal perforation secondary to esophageal foreign body in dogs. *J Am Vet Med Assoc,* 253 (8): 1053-1056, 2018. DOI: 10.2460/javma.253.8.1053

Kafkas Univ Vet Fak Derg
26 (2): 217-224, 2020
DOI: 10.9775/kvfd.2019.22767

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Effect of β-glucanase on Performance, Carcass Characteristics, Microflora, Plasma Constitutes and Immunity in Local Broiler Hybrid "Golpayegani-Ross"

Mohammadebrahim TASIRNAFAS [1,a] Kazem KARIMI [1,b] Ghobad ASGARI JAFARABADI [1,c]
Fatemeh NOORBAKHSH [2,d] Alireza SEIDAVI [3,e]

[1] Department of Animal Science, Faculty of Agriculture, Varamin-Pishva Branch, Islamic Azad University, Varamin-Pishva, IRAN
[2] Department of Microbiology, Biological Science College, Varamin-Pishva Branch, Islamic Azad University, Varamin-Pishva, IRAN
[3] Department of Animal Science, Rasht Branch, Islamic Azad University, Rasht, IRAN
[a] ORCID: 0000-0002-5272-9819; [b] ORCID: 0000-0001-5318-2512; [c] ORCID: 0000-0002-7192-1558; [d] ORCID: 0000-0001-8082-8446
[e] ORCID: 0000-0002-1903-2753

Article ID: KVFD-2019-22767 Received: 30.05.2019 Accepted: 07.11.2019 Published Online: 07.11.2019

How to Cite This Article

Tasirnafas M, Karimi K, Asgari Jafarabadi G, Noorbakhsh N, Seidavi A: Effect of β-glucanase on performance, carcass characteristics, microflora, plasma constitutes and immunity in local broiler hybrid "Golpayegani-Ross". *Kafkas Univ Vet Fak Derg,* 26 (2): 217-224, 2020.
DOI: 10.9775/kvfd.2019.22767

Abstract

The Gram negative bacterium *Fibrobacter succinogenes* lives anaerobically in the bovine rumen and secretes β-glucanase. Barley is a cheap food input for poultry production but it is low in nutrients due to its beta glucan content. Supplementing exogenous β-glucanase (EC 3.2.1.4) to broiler diets can decrease the viscosity of the intestinal content by hydrolyzing the β-glucan and enhancing nutrient digestibility. In the current study, β-glucanase enzymatic activity was assayed after extraction from bovine rumen fluid, and then its effect on broiler performance, carcass characteristics, duodenum microbial flora, hematological and immunological parameters was compared with a commercial enzyme. A total of 120 local broilers (Golpayegani-Ross hybrid) was allocated to 3 treatments with 4 replicates per treatment and 10 birds per replicate/pen. Over a 49-day experimental period, broilers were fed a basal diet (T1), basal diet with 10 IU of extracted β-glucanase and 20% barley (T2) and basal diet with 10 IU of commercial β-glucanase and 20% barley (T3). T2 significantly increased body weight gain and decreased feed intake over the whole experimental period. Treatments had a significant effect on hematological parameters except low density lipoprotein (LDL) concentration ($P<0.01$). Treatments did not affect antibody titration. The highest non-eviscerated carcass weight ($P<0.05$) and eviscerated carcass weight ($P>0.05$) were associated with the T2. The same treatment also caused a significant increase in lactobacilli and *Escherichia coli* in the gastrointestinal tract. Consequently, enzyme addition had a positive effect on broiler performance without any adverse effects on humoral immunity parameters.

Keywords: *Broiler performance, Carcass characteristics, Enzymatic extraction, Fibrobacter succinogenes, Microbial flora*

Lokal Broyler Hibridi "Golpayegani-Ross"da Performans, Karkas Karakteristiği, Mikroflora, Plazma Bileşenleri ve Bağışıklık Üzerine β-glukanazın Etkisi

Öz

Gram negatif bakteri *Fibrobacter succinogenes*, sığır rumeninde anaerobik olarak yaşar ve β-glukanaz salgılar. Arpa, kanatlı hayvan üretimi için ucuz bir besin maddesidir, ancak içerdiği beta glukan nedeniyle besin değeri düşüktür. Broyler diyetlerine eksojen β-glukanazın (EC 3.2.1.4) eklenmesi, β-glukanın hidrolize edilmesi yoluyla intestinal içeriğin viskozitesini azaltarak besinlerin sindirilebilirliğini arttırabilir. Bu çalışmada, sığır rumen sıvısından yapılan ekstraksiyondan elde edilen β-glukanazın enzimatik aktivitesi incelendi ve daha sonra broyler performansı, karkas özellikleri, duodenum mikrobiyal florası, hematolojik ve immünolojik parametreler üzerindeki etkisi ticari bir enzimle karşılaştırıldı. Toplam 120 lokal ırk broyler (Golpayegani-Ross hibrid), uygulama başına 4 tekrar ve her tekrarda 10 hayvan olacak şekilde 3 gruba ayrıldı. Kırkdokuz günlük deney süresince, broylerlere bazal diyet (T1), 10 IU ekstrakte β-glukanaz ve %20 arpa içeren bazal diyet (T2) ve 10 IU ticari β-glukanaz ve %20 arpa içeren bazal diyet (T3) verildi. Tüm deney dönemi boyunca T2 diyeti vücut ağırlığını önemli ölçüde arttırdı ve yem alımını azalttı. Tedaviler, düşük yoğunluklu lipoprotein (LDL) konsantrasyonu ($P<0.01$) dışındaki hematolojik parametreler üzerinde önemli bir etkiye sahipti. Bununla birlikte antikor düzeyi farklı tedavilerden etkilenmedi. İç organları uzaklaştırılmamış ($P<0.05$) ve iç organları uzaklaştırılmış en yüksek karkas ağırlığı ($P>0.05$) T2 diyeti ile beslenen grupta belirlendi. Aynı tedavi gastrointestinal sistemde laktobasil ve *Escherichia coli* düzeyinde de önemli bir artışa neden oldu. Sonuç olarak enzim ilavesi, humoral bağışıklık parametreleri üzerinde herhangi bir olumsuz etkisi olmaksızın, broyler performansını olumlu etkilemiştir.

Anahtar sözcükler: *Broyler performansı, Karkas özellikleri, Enzimatik ekstraksiyon, Fibrobacter succinogenes, Mikrobiyal flora*

İletişim (Correspondence)
+98 919 4449056
karimikazem@gmail.com

INTRODUCTION

The *Fibrobacter succinogenes*, an obligate anaerobic bacterium, is an important degrader of lignocellulosic plant material in the herbivore gastrointestinal tract. Enzymes produced by *F. succinogenes* have the cellulases, endoglucanases and xylanases, which were thought to be involved in cellulose degradation. These membrane vesicles found in *F. succinogenes* cellulose cultures have not a role in cellulose degradation, but they are sign of aging cells [1]. Gong et al.[2] discovered a 180 kDa cellulose binding protein with a role in adhesion to cellulose. Most anaerobic cellulose degrading bacteria rely upon strict binding of the cell to the cellulose fiber. Hence, based on this discovery is proposed the binding proteins termed "fibro-slime" proteins. These proteins are specific to *F. succinogenes* [1].

Corn is the main material used in poultry diets due to its high energy and lack of antinutritive effects but there is competition for consumption of cereals like corn between humans and monogastric animals [3]. There is also interest in replacing corn with other materials, such as barley in diets, due to restricted culture of corn [4].

The use of commercial enzymes in poultry nutrition is of great importance. A proportionate increase in feed ingredients price has been the primary impediment in almost all developing countries. As a result, non-conventional and cheaper feed ingredients should be used which have a higher percentage of non-starch polysaccharides (NSPs) along with starch [5].

To promote growth, protect well-being and maximize the genetic potential of modern broiler and layer hybrids, growth promoting feed additives have been commonly included in poultry diets [6]. The gel-forming characteristic of β-glucan after water absorption is similar to that of other NSPs like pectin and gum arabic [7]. Feeding birds with barley is problematic because of viscosity and adhesivity due to presence of β-glucans during digestion. Supplementing broiler diets with exogenous β-glucanase (EC 3.2.1.4) can decrease the viscosity of the intestinal content by hydrolyzing the β-glucan and enhance nutrient digestibility [7]. B-glucans account for up to 70% of the cell wall in the barley endosperm and β-glucanase can be a valuable tool as a natural way to enhance β-glucan degradation and to improve feed utilization [8].

We know that cereals with high levels of non-starch polysaccharides, like barley, cause electrolytic imbalance of diet. Adding β-glucanase, which can by directly mediate absorption and transmission of minerals through receptors situated on the intestine luminal wall, can prevent acidification of intestine and body fluids, reduce negative effects of body homeostasis imbalance and improve broiler growth and performance through accelerating absorption function of the intestine [9].

Wang et al.[10] investigated the effects of exogenous enzyme supplementation to diets on the growth performance and nutrient digestibility in broiler chickens.

These authors reported that supplementing diets with enzymes can increase digestibility, reduce diet cost, improve growth surface properties and feed efficiency in addition to reducing organic wastes [10]. They also reported that accuracy in selecting enzymes can well improve performance in poultry and supplementing diet with enzyme, apart from economic advantages, can influence environmental conditions and improve diet particles [10]. For investigation of gastrointestinal microbial flora, caecum contents were sampled at the age of 42 days [10].

The aims of current study were to:

a- Extract and purify β-glucanase from the bovine rumen bacterium *Fibrobacter succinogenes*

b- Investigate the effect of supplementation of diets for a local broiler hybrid Golpayegani-Ross with the extracted enzyme on performance, carcass characteristics,

c- Investigate the effect of supplementation of diets for a local broiler hybrid Golpayegani-Ross microbial flora, plasma biochemical parameters and immunity.

MATERIAL and METHODS

Bovine Rumen Fluid

This study started on 22 August 2017 at the microbiology laboratory of the agriculture faculty of Islamic Azad University, Varamin-Pishva Branch, Varamin, Iran. Bovine rumen fluid was collected from a slaughterhouse in Varamin, Iran into sterile tubes and transferred to the microbiology laboratory. Approximately 1 L of rumen fluid was collected, centrifuged for 30 min at 4°C and 1.000 rpm. The transparent supernatant was stored at -20°C.

Preparation of Fibrobacter succinogenes Culture Medium

The composition of the culture medium is shown in *Table 1*.

The solution was heated under vacum to boiling point. Then, the solution was put under CO_2 100% pressure and cooled. Sodium carbonate was then added and the solution was boiled for a further 10 min. The solution was autoclaved with two other separately prepared solutions, 10 mL L-Cysteine. HCl 2.5% and 10 mL $Na_2S.9H_2O$ for 15 min at 121°C. After autoclaving and cooling, the two other solutions (10 mL L-Cysteine. HCl 2.5% and 10 mL $Na_2S.9H_2O$) were added to the main solution. The final pH was set at 6.6±0.1, and the solution was distributed in previously sterilised 50 mL containers. The rumen fluid was injected using a 50 µL sampler. All containers were maintained in 34°C incubator for 2 weeks. The tubes were then taken out and centrifuged at 1.400 rpm for 30 min. Then, 20 µL supernatant was decanted by sampler on microscope lamella and stained for microscopy as explained below:

Table 1. *The composition of theculture medium for bovine rumen bacterium Fibrobacter succinogenes*

Number	Content	Amount
1	KH_2PO_4	0.3 g
2	K_2HPO_4	0.3 g
3	$(NH_4)_2SO_4$	0.3 g
4	NaCl	0.6 g
5	$MgSO_4.7H_2O$	0.12 g
6	$CaCl_2.2H_2O$	0.08 g
7	Tryptone (BD 211705)	1.0 g
8	Yeast extract	0.5 g
9	Resazurin	1.0 mg
10	Cellobiose	4.0 g
11	Vitamin solution[1]	20.0 mL
12	Trace elements[2]	1.0 mL
13	VFA solution[3]	4.65 mL
14	Na_2CO_3	4.0 g
15	Distilled water	960.0 mL

[1] Vitamin solution includes:Lipoic acid, 20.0 mg; Thiamine.HCl, 20.0 mg; Calcium D-(+)-pantothenate, 20.0 mg; Nicotinamide, 20.0 mg; Riboflavin, 20.0 mg; Pyrodoxal hydrochloride 20.0 mg; Pyridoxamine.2HCl, 20.0 mg; p-aminobenzoic acid 1.0 mg; Biotin, 1.0 mg; Cyanocobalamin, 1.0 mg; Distilled water, 100.0 mL; [2] Trace elements include: $ZnSO_4.7H_2O$, 0.1 g; $MnCl_2.4H_2O$, 0.03 g; H_3BO_3 0.2 g; $COCl_2.6H_2O$ 0.2 g; $CuCl_2.2H_2O$ 0.01 g; $NiCl_2.6H_2O$ 0.02 g; $Na_2MoO_4.2H_2O$, 0.03 g; $FeSO_4.7H_2O$ 2.0 g; Distilled water, 1.0 L; [3] Volatile Fatty Acids include: Acetic acid, 17.0 mL; Propionic acid, 6.0 mL; n-Butyric acid, 4.0 mL, n-Valeric acid, 1.0 mL, IsoValeric acid, 1.0 mL, IsoButyric acid, 1.0 mL, DL-alpha-methylbutyric acid, 1.0 mL, Distilled water, 310.0 mL

For preparing smears, 20 µL supernatant was removed from 50 µL containers and put on lamella. Then, crystal violet was poured on lamella for 1-2 min, and washed with distilled water. Then, Lugol's solution was poured for 1 min and washed with distilled water. Ethylic alcohol was poured and washed with distilled water. Safranin was poured for 30 sec to 1 min and washed with distilled water. 100X microscopy was used for identification of bacterium *Fibrobacter succinogenes*.

Enzyme Extraction and Purification

Briefly, 8.7 g potassium phosphate (K_2HPO_4) and 2.5 g citric acid ($C_6H_8O_7$) were mixed with 1 L distilled water to a final pH equal to 6.5.

Substrate Solution

Briefly, 7.5 g β-glucan solution was mixed with 500 mL citrate-phosphate buffer and autoclaved at 121°C for 15 min.

Primary 3,5-Dinitrosalicylic Acid (DNS) Solution

Briefly, 5 g 3,5-dinitrosalicylic acid, 1 g phenol and 5 g sodium hydroxide were dissolved in 500 mL distilled water.

Sodium Phosphate Solution 5%

Briefly, 1 g sodium phosphate (Na_2HPO_4) was dissolved in 20 mL distilled water.

Glucose Solution 5%

Briefly, 1 g glucose was dissolved in 20 mL distilled water.

Secondary 3,5-Dinitrosalicylic Acid (DNS) Solution

Briefly, 0.5 mL sodium phosphate (Na_2HPO_4) and 20 µL glucose solution was completely mixed with 50 mL of primary DNS solution.

Standard Glucose Preparation

The solution was prepared by mixing glucose with distilled water (1 $mg.mL^{-1}$). Briefly, 1080 µL β-glucanase substrate solution was poured into a 1.5-mL microtube and 180 µL centrifuged rumen liquid was added to it. The microtubes were maintained at 37°C in a water bath for 30 min. The microtubes were then removed and the reaction terminated by addition of 1440 µL 3,5-dinitrosalicylic acid. The microtubes were subsequently placed in warm water for staining and after 20 min, the microtubes were removed and immediately cooled. Absorption was measured by a spectrometer at 570 nm. To determine the relationship between absorption and concentration, a standard curve was developed. One $mg.mL^{-1}$ glucose solution was added to the microtubes in volumes 0, 72, 144, 216, 288 and 360 µL. Dinitrosalicylic acid was subsequently added to reach a volume of 2880 µL. The absorption values for samples and standards were read at a wavelength of 570 nm. After reading the absorption of the glucose standard, the concentration was calculated by the formula below:

$y = a + bx$. Enzyme activity is shown in the *Fig. 1*.

According to the calculations, an enzymatic activity of 0.382 IU was observed per g of *Fibrobacter succinogenes*culture medium *(Fig. 1* and *Table 1)*. Consequently, the enzymatic activity of 5.25 g of *Fibrobacter succinogenes*culture equals 2 IU *(Fig. 1)*.

Treatments and Experimental Diets

In this experiment, 120 one-day-old local hybrid broiler chicks (Golpayegani-Ross hybrid) were transferred to the Naranj-Gol Poultry Farm, Rasht, Iran (37.2682° N, 49.5891° E). The experimental protocol was ratified by the Animal Ethic Committee of the Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran, and the experiment was performed with respect to the International Guidelines for research involving animals (Directive 2010/63/EU).

Broilers were randomly allocated to 3 treatments with four replicates/pensper treatment and 10 birds per pen. Chickens were raised under controlled temperature and air condition and continuous 24 h light. Each pen was equipped with a handle pan feeder and a manual drinker. During the whole experiment, water was provided *ad libitum*. The experiment lasted for 49 days and vaccination programs were done regularly.

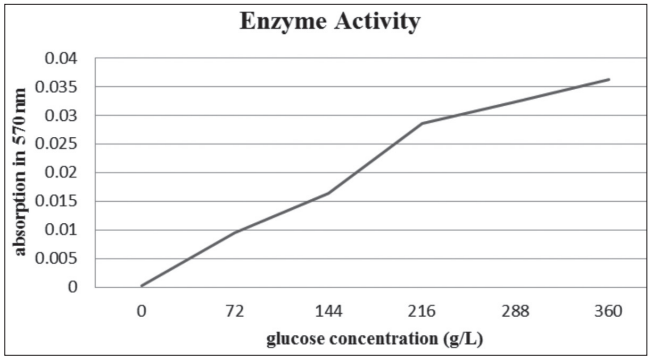


Fig 1. Standard absorption of glucose graph, horizontal axis shows glucose concentration (mg/mL) and vertical axis shows enzyme activity ($mg.min^{-1}.mL^{-1}$)

acid, triglycerides, HDL, HDL cholesterol ratio, LDL, LDL cholesterol ratio, alkaline phosphatase, IgG1, IgG2, IgM1 and IgM2. Tagged birds were injected with 0.2 mL diluted sheep blood (SRBC) and their blood samples were used for hematological tests. At the end of the experiment, 2 birds from each treatment were randomly selected for determination of carcass characteristics including featherless carcass weight, abdomen full carcass weight, eviscerated carcass weight, head weight, breast weight, thigh weight, abdominal fat weight, and neck weight. Microbial flora including anaerobic bacteria, aerobic bacteria, lactobacilli and *Escherichia coli* were counted.

For counting bacteria, colony forming unit method was used. For this purpose, 1 g of caecum contents were added

Table 2. *Ingredient composition of the experimental rations*

Treatments	T1[1]			T2[1]			T3[1]		
	St[2]	Gr[3]	Fn[4]	St[2]	Gr[3]	Fn[4]	St[2]	Gr[3]	Fn[4]
Enzyme (IU)	0	0	0	10 IU Fib.[5]	10 IU Fib.[5]	10 IU Fib.[5]	10 IU com.[6]	10 IU com.[6]	10 IU com.[6]
Barley	20	20	20	20	20	20	20	20	20
Corn	44.675	47.86	51.535	44.675	47.86	51.535	44.675	47.86	51.535
Soybean	30.889	27.045	22.848	30.889	27.045	22.848	30.889	27.045	22.848
Soybean oil	0.238	1.126	1.884	0.238	1.126	1.884	0.238	1.126	1.884
DL-methionine	0.295	0.253	0.236	0.295	0.253	0.236	0.295	0.253	0.236
L-lysine HCl	0.235	0.205	0.211	0.235	0.205	0.211	0.235	0.205	0.211
L-threonine	0.13	0.074	0.068	0.13	0.074	0.068	0.13	0.074	0.068
Di calcium-phosphate	1.783	1.688	1.482	1.783	1.688	1.482	1.783	1.688	1.482
$CaCO_3$	0.804	0.79	0.739	0.804	0.79	0.739	0.804	0.79	0.739
Na-bBicarbonate	0.266	0.265	0.399	0.266	0.265	0.399	0.266	0.265	0.399
NaCl	0.185	0.194	0.098	0.185	0.194	0.098	0.185	0.194	0.098
Vitamin[7] and mineral premix[8]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

[1] basal diet included 20% barley (T1), basal diet included 10 IU extracted β-glucanase and 20% barley (T2) and basal diet included 10 IU, commercial β-glucanase and 20% barley (T3); [2] Starter period (1-14 days); [3] Grower period (15-35 days); [4] Finisher period (36-49 days); [5] β-glucanase enzyme extracted from wild strain F. succinogenes; [6] commercial β-glucanase enzyme; [7] Each kilogram contains: 5.000.000 IU Vit. A, 2.000.000 IU Vit. D_3, 32.000 mg Vit. E, 1.280 mg Vit. K_3, 1.274 mg Vit. B_1, 3.440 mg Vit. B_2, 25.000 mg Vit. B_3, 7.416 mg Vit. B5, 1.944 mg Vit. B_6, 880 mg Vit. B_9, 8 mg Vit. B_{12}, 100 mg Vit. Biotin H_2, Antioxidant 1000 mg; [8] Each kilogram contains: 48.018 mg Mn, 8.092 mg Fe, 44.030 mg Zn, 6.448 mg Cu, 501 mg I, 121 mg Se

The treatments were as follows:

T1 (control): Corn and soybean meal without supplemental enzyme and barley

T2: Corn and soybean meal with 20% barley and 10 IU β-glucanase enzyme extracted from *Fibrobacter succinogenes* (per kg diet)

T3: Corn and soybean meal with 20% barley and10 IU commercial β-glucanase enzyme (Rovabio™) (per kg diet)

Feed ingredients and nutrient percentages are shown in *Table 2* and *Table 3*.

Measured Parameters

Body weight gain and feed consumption were measured during the experiment. The hematological parameters measured were fasting blood sugar (FBS), cholesterol, uric

to 9 mL of phosphate buffer and then serial 10-fold dilutions were prepared. In order to count *Lactobacilli*, sharp agar deman-rogosa was used as the culture medium. To count total anaerobic bacteria, incubation was performed in anaerobic jars for 72 h. For total aerobic bacteria, *Escherichia coli* was extracted from nutrient agar and eosin methylene blue agar aerobically for 24 h [11,12].

On day 20, from each pen 2 birds were tagged and 2 mL defibrinated sheep blood plus 47.5 mL physiologic serum prepared for SRBC were injected intothe breast muscle. One week later, blood was taken from the tagged birds for laboratory tests. Total serum antibody titres to SRBC were determined by hemaglutination assay. For investigating immune response, all birds were vaccinated for Newcastle disease virus (NDV) through drinking water and one week later, blood was taken from 2 birds from each pen and appropriate immunological tests were performed.

Table 3. *Chemical composition of the experimental rations*

Treatment	T1[1]			T2[1]			T3[1]		
	St[2]	Gr[3]	Fn[4]	St[2]	Gr[3]	Fn[4]	St[2]	Gr[3]	Fn[4]
Enzyme (IU)	0	0	0	10 IU Fib.[5]	10 IU Fib.[5]	10 IU Fib.[5]	10 IU com.[6]	10 IU com.[6]	10 IU com.[6]
Dry matter (%)	89.328	89.323	89.299	89.328	89.323	89.299	89.328	89.323	89.299
Crude protein (%)	19.624	18.092	16.527	19.624	18.092	16.527	19.624	18.092	16.527
Energy (ME) (Kcal.kg^{-1})	2800	2900	3000	2800	2900	3000	2800	2900	3000
Calcium (%)	0.840	0.800	0.720	0.840	0.800	0.720	0.840	0.800	0.720
Available phosphorus (%)	0.420	0.400	0.360	0.420	0.400	0.360	0.420	0.400	0.360
Sodium (%)	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160	0.160
Lysine (%)	1.1	0.990	0.900	1.1	0.990	0.900	1.1	0.990	0.900
Methionine (%)	0.555	0.498	0.464	0.555	0.498	0.464	0.555	0.498	0.464
Methionine and cysteine (%)	0.825	0.752	0.702	0.825	0.752	0.702	0.825	0.752	0.702

[1] Basal diet included 20% barley (T1), basal diet included 10 IU extracted β-glucanase and 20% barley (T2) and basal diet included 10 IU commercial β-glucanase and 20% barley (T3); [2] Starter period (1-14 days); [3] Grower period (15-35 days); [4] Finisher period (36-49 days)

Data Analysis

The study was conducted using 120 chicks in a completely randomized design with 3 treatments and 4 replicates. The experimental unit was the pen. The results of the study were subjected to one-way analysis of variance (ANOVA) using the GLM procedure of SPSS v 24 for windows [13]. Differences in the experimental treatments were tested using Duncan's Multiple Range Test following ANOVA with significance reported at P≤0.05.

RESULTS

BWG, FI and FCR did not differ between treatments in the starter period, but their effects were significant for BWG in finisher (P>0.05) and whole period (P<0.01), and FCR in whole period (P<0.01). In whole period, the highest BWG (16.2±0.5), the lowest FI (36.3±0.4) and the lowest FCR (2.2±0.1) were all associated with T2 *(Table 4)*.

In the current study, T2 resulted in the lowest FI for whole period but this was not significant (P>0.01). The best FCR in the grower (P<0.01), finisher (P>0.05) and whole periods (P>0.05) was associated with T2 *(Table 4)*.

In the present study, the effects of different treatments were significant for all hematological parameters, except LDL (P<0.01) *(Table 5)*. The highest concentrations of blood cholesterol, HDL and LDL were associated with T2, while the highest concentration of FBS, uric acid, triglycerides, and alkaline phosphatase were associated with T3 (basal diet and 10 IU commercial β-glucanase) (P<0.01).

Treatments did not have a significant effect on antibody titration *(Table 6)*. The highest concentrations of IgG1, IgG2 and IgM1 were associated with T3 (P>0.05) *(Table 6)*.

The highest non-eviscerated carcass weight (P<0.05) and eviscerated carcass weight (P>0.05) belonged to T2 *(Table 7)*. The highest head weight (P<0.05), breast weight (P>0.05), tight weight (P<0.01) and abdominal fat weight (P<0.05) were associated with the T2 *(Table 7)*.

T2 resulted in a significant increase of lactobacilli and *Escherichia coli* in broilers' duodenum (P<0.05) *(Table 8)*.

DISCUSSION

Poultry do not produce enzymes that hydrolyze the NSPs in the cell wall if grains which remain without decomposition and cause a reduction in feed efficiency. Supplementation with preparations of exogenous enzymes in the diet is one approach to overcoming the adverse effects of NSPs. This has a positive effect on feed digestibility and leads to better productivity and performance [5].

Our results contrast with those of Agah et al.[14] who reported that enzyme supplementation had no significant effect on BWG and FI in comparison with an unsupplemented control in the starter, growth and whole periods. The basal diet with 10 IU extracted β-glucanase and 20% barley (T2) resulted in a significant increase in BWG for the whole period (P<0.01). This is in agreement with the finding of Abudabos [15] who reported that enzyme supplementation significantly increased body weight at 42 and 49 d of age. The strain used in that study was Cobb 400 while the strain used in current study was Golpayegani-Ross hybrid. The enzyme used in that study was Bergazyme, a commercial enzyme supplement that contains β-pentosanase, α-amylase, β-glucanases, glucanases, and galactomannases.

Our results are in agreement with Mathlouthi et al.[16] who reported that during the whole experimental period (1-40 d) FCR was decreased (P≤0.05) by β-glucanase supplementation. Our results are however, in inconsistent with those of Kalantar et al.[17] who reported that supplementing wheat and barley diets with multi-enzymes increased total feed intake and decreased feed conversion ratio significantly (P<0.05) compared to non supplementation. Moreover, our results are inconsistent

Table 4. *BWG, FI and FCR in chicks fed the experimental rations; means ± standard deviation*

Items	Experimental Periods	T1[1]	T2[1]	T3[1]	P-value	SEM
Body weight gain (g/bird/day)	Starter (1-14d)	15.6±0.8[a]	16.8±1.1[a]	16.1±0.5[a]	0.19	0.27
	Grower (15-35d)	13.2±1.6[a]	12.7±1.1[a]	13.7±0.7[a]	0.41	0.35
	Finisher (36-49d)	14.0±2.4[a]	20.9±3.8[a]	15.8±3.4[a]	0.03	1.22
	Whole (1-49d)	14.1±0.7[b]	16.2±0.5[a]	15.0±0.6[ab]	0.00	0.30
Feed intake (g/bird/day)	Starter (1-14d)	19.1±1.1[a]	20.8±1.1[a]	20.4±1.4[a]	0.18	0.38
	Grower (15-35d)	37.3±0.7[ab]	36.3±0.3[b]	38.0±0.5[a]	0.00	0.25
	Finisher (36-49d)	53.2±2.0[a]	50.6±0.5[a]	52.2±1.5[a]	0.08	0.50
	Whole (1-49d)	37.0±0.7[a]	36.3±0.4[a]	37.4±0.6[a]	0.07	0.21
Feed conversion ratio (g/g)	Starter (1-14d)	1.2±0.1[a]	1.2±0.1[a]	1.3±0.0[a]	0.67	0.02
	Grower (15-35d)	2.9±0.4[a]	2.9±0.3[a]	2.7±0.1[a]	0.76	0.08
	Finisher (36-49d)	3.9±0.8[a]	2.5±0.5[a]	3.4±0.8[a]	0.05	0.25
	Whole (1-49d)	2.6±0.1[a]	2.2±0.1[b]	2.5±0.1[a]	0.00	0.05

[a-c] Means within each row with no common superscripts differ significantly at P≤0.05; [1] basal diet (T1), basal diet included 10 IU extracted β-glucanase (T2) and basal diet included 10 IU commercial β-glucanase (T3)

Table 5. *Hematological parameters in chicks fed the experimental rations; means ± standard deviation*

Parameters	T1[1]	T2[1]	T3[1]	P-value	SEM
FBS[2] (mg/dL)	1.5±0.1[c]	2.1±0.1[b]	2.4±0.1[a]	<0.001	2.01
Cholesterol (mg/dL)	142.3±1.0[b]	149.5±1.9[a]	125.3±1.0[c]	<0.001	3.08
HDL[3] (mg/dL)	92.00±0.8[b]	97.5±1.3[a]	76.0±0.8[c]	<0.001	2.76
HDL/cholesterol ratio	1.6±0.0[a]	1.4±0.1[b]	1.5±0.1[ab]	<0.001	0.02
LDL[4] (mg/dL)	37.8±1.50[b]	42.5±1.9[a]	34.0±1.6[b]	<0.001	1.13
LDL/cholesterol ratio	0.4±0.1[b]	0.4±0.0[a]	0.4±0.1[a]	0.06	0.01
Triglyceride (mg/dL)	62.8±1.0[b]	64.5±1.3[a]	70.8±2.2[a]	<0.001	4.70
Alkaline phosphatase (U/L)	5055±12.9[b]	5472.5±17.1[a]	5477.5±12.6[a]	<0.001	59.81
Uric acid (mg/dL)	1.5±0.1[c]	2.1±0.1[b]	2.4[a]±0.1	<0.001	0.12

[a-c] Means within each row with no common superscripts differ significantly at P≤0.05; [1] basal diet (T1), basal diet included 10 IU extracted β-glucanase (T2) and basal diet included 10 IU commercial β-glucanase (T3); [2] Fasting blood sugar; [3] High Density Lipoprotein; [4] Low Density Lipoprotein

Table 6. *Immunological parameters (log 10) in chicks fed the experimental rations; means ± standard deviation*

Parameters	T1[1]	T2[1]	T3[1]	P-value	SEM
Immunoglobulin G1	1.8±1.0[a]	2.0±1.4[a]	2.5±1.0[a]	0.65	0.31
Immunoglobulin G2	3.0±1.4[a]	3.0±0.8[a]	4.0±1.4[a]	0.45	0.35
Immunoglobulin M1	3.0±1.4[a]	2.0±1.2[a]	3.3±1.0[a]	0.33	0.35
Immunoglobulin M2	2.5±0.6[a]	3.3±1.0[a]	2.5±0.6[a]	0.29	0.21

[a-c] Means within each row with no common superscripts differ significantly at P≤0.05; [1] basal diet (T1), basal diet included 10 IU extracted β-glucanase (T2) and basal diet included 10 IU commercial β-glucanase (T3)

with those of Zou et al.[18] who reported there were no significant difference in BWG and FI of broilers for enzymes BM (a commercial β-mannanase product), AG (a commercial galactosidase product), and XG (a commercial product containing endo-xylanase and β-glucanase).

In the current study, adding enzymes had a significant positive effect on performance. Enzymes can decrease viscosity and enhance feed digestibility and feed intake.

Since β-glucans are structural carbohydrates and have high molecular weight, they exist in soluble form in small intestine [19]. Therefore, intestinal contents are in viscous soluble form, which prevents free moving of other soluble contents [19]. Under such conditions, water absorption capacity increases in the intestine, which in turn, increases the viscosity of the contents and ultimately restricts nutrient absorption [19].

Studies have shown that adhesive effects of β-glucans can be mitigated through enzymatic hydrolysis by β-glacanases [20]. Enzymes are able to hydrolyze adhesive polymers (soluble non-starch polysaccharides) and convert them into small polymers through complete hydrolysis thereby decreasing viscosity of digesta and releasing nutrients encapsulated by these viscous compounds [20].

Table 7. *Carcass characteristicsin chicks fed the experimental rations; means ± standard deviation*

Items	T1[1]	T2[1]	T3[1]	P-value	SEM
Featherless carcass (g)	559.50±17.6[a]	624.0±58.9[a]	536.5±72.0[a]	0.11	18.11
Non-eviscerated carcass (g)	486.4±6.2[b]	559.0±46.8[b]	471.0±67.0[a]	0.05	16.92
Eviscerated carcass (g)	289.0±97.0[a]	371.8±34.8[a]	298.5±37.2[a]	0.18	19.92
Dressing percentage (%)	42.7±14.2[a]	47.8±4.2[a]	41.4±4.9[a]	0.58	2.50
Head (g)	26.5±1.5[a]	29.2±1.8[ab]	27.1±3.5[a]	0.01	0.72
Breast (g)	80.1±1.2[a]	85.1±8.4[a]	75.2±14.3[a]	0.38	2.78
Tight (g)	117.2±4.8[b]	149.2±18.1[a]	112.0±15.4[b]	<0.00	6.15
Abdominal fat (g)	8.6±3.7[b]	20.9±5.5[a]	12.7±5.9[ab]	0.02	2.04

[a-c] Means within each row with no common superscripts differ significantly at P≤0.05; [1] basal diet (T1), basal diet included 10 IU extracted β-glucanase (T2) and basal diet included 10 IU commercial β-glucanase (T3)

Table 8. *Deodenal bacteria (log CFU/mL) in chicks fed the experimental rations; means ± standard deviation*

Items	T1[1]	T2[1]	T3[1]	P-value	SEM
Aerobic bacteria	6.6±0.3[a]	6.7±0.4[a]	7.1±0.3[a]	0.14	0.10
Lactobacilli	7.3±0.0[a]	7.6±0.3[a]	7.1±0.3[a]	0.03	0.08
Anaerobic bacteria	6.7±0.3[a]	7.0±0.3[a]	7.0±0.3[a]	0.31	0.08
Escherichia coli	6.5±0.2[b]	7.1±0.3[a]	6.5±0.1[b]	0.002	0.10

[a-c] Means within each row with no common superscripts differ significantly at P≤0.05; [1] basal diet (T1), basal diet included 10 IU extracted β-glucanase (T2) and basal diet included 10 IU commercial β-glucanase (T3)

Viscosity increases small intestine length and weight in different ways. First, NSP compositions, by stretching the intestine wall, apply pressure to the wall and its muscular layers, which causes an increase in sarcomere length and the muscular layer myofibrils to combat the applied pressure. Moreover, an increase in the length and width of intestinal wall muscles is an adaptation mechanism by the bird to make viscous materials move in its intestine. The second mechanism by which viscosity increases intestine length is probably bird need to nutrients [5]. The beneficial effects of enzyme addition are probably associated with an increase in nutrient consumption through decreasing intestine viscosity and removing anti-nutritional effects of non-starch polysaccharides [5].

In the case of the triglyceride content our findings comply with those of Abudabos [15] who reported an increase of broilers' triglyceride concentration due to enzyme supplementation.

It has been shown that β-glucan can enhance glucose and insulin hemostasis and decrease blood cholesterol level [21]. For this reason, it can be proposed that in the current study enzyme addition results in an increase in blood cholesterol level through digesting β-glucan content.

According to our results, cholesterol content for treatment 2 was higher than in treatment 3. Serum cholesterol is affected by diet and factors, such as non-starch polysaccharides, can have an effective role in this matter.

Nahas and Lefrancois [22] stated that NSPs in barley are effective in transferring and metabolism of lipids and can reduce blood cholesterol level. Increase of NSP levels in diet causes decrease of lipid digestion and probably destroys cholesterol reabsorption cycle through adhesivity and decreases in blood cholesterol. Moreover, short chain fatty acids resulting from NSP digestion in large intestine can stop cholesterol synthesis through excretion of bile acids and secretion of natural steroids [23].

Cholesterol increase in the group supplemented with enzyme extracted from *Fibrobacter suuccinogenes*, apart from its high purity, increases digestibility, decreases viscosity of digesta in gastrointestinal tract and improves better digestion of it including fatty acids [24]. As a result, cholesterol content is increased in birds consuming enzyme additive extracted from *Fibrobacter suuccinogenes* in comparison with the control group and the third group.

Our results are in agreement with those of Basmacioğlu Malayoğlu et al.[25] who reported that enzyme supplementation had no significant effect on immune response of broilers fed on wheat–soybean meal diets. Our results agree with those of Khaksar et al.[26] who reported antibody production against sheep red blood cell (SRBC) antigen and other haematological analysis were numerically (P>0.05) enhanced in broilers fed wheat-based diet with the addition of Endofeed W enzyme, containing arabinoxylanase and β-glucanse. Seidavi et al.[27] reported significant increase of IgG in broilers fed enzyme and probiotic supplemented diets after the second challenge with SRBC (P<0.01). Changes observed in immune response may be attributed to changes in broilers' intestinal microbial population since microorganisms naturally living in the intestinal tract are crucial for poultry digestion and immunity [27].

Our results are in agreement with those of Agah et al.[14] in that enzyme supplementation had no significant effect on breast weight and tight weight. In the case of dressing percentage, our results are in agreement with Abudabos [15] who reported that this parameter was slightly increased by enzyme supplementation. He suggested that the difference in dressed yield of broilers could be due to the different diets and kind and level of enzyme used [15]. In the case of abdominal fat weight, our results are in agreement with those of Agah et al.[14] and Mathluthi et al.[16] who reported that abdominal fat weight was not affected by the addition of enzyme in broiler feed.

Our results contrast with those of Mathluthi et al.[16] who reported a decrease in the number of *E. coli* in the caeca of broilers due to the addition of xylanase and β-glucanase. Khaksar et al.[26] reported increase in ileal microbial population of Lactobacillus and decrease in ileal microbial population of *E. coli*.

It is concluded that the basal diet with 10 IU extracted β-glucanase and 20% barley (T2) resulted in a significant increase in body weight gain and decreased feed intake over the whole experimental period. This treatment enhanced FCR over the whole experimental period (P<0.05).

REFERENCES

1. Burnet MC, Dohnalkova AC, Neumann AP, Lipton MS, Smith RD, Suen G: Evaluating models of cellulose degradation by *Fibrobacter succinogenes* S85. *PLoS ONE,* 10 (12): e0143809, 2015. DOI: 10.1371/journal.pone.0143809

2. Gong JH, Egbosima EE, Forsberg CW: Cellulose binding proteins of *Fibrobacter succinogenes* and the possible role of a 180-KDa cellulose-binding glycoprotein in adhesion to cellulose. *Can J Microbiol*, 42 (5): 453-460, 2011. DOI: 10.1139/m96-062

3. Shahir MH, Moradi S, Afsarian O, Heydarinia A: Effect of adding enzyme and organic acid in corn and wheat based diets on performance and intestinal morphological characteristics of broiler chicken. *Iranian J Anim Sci Res,* 3 (4): 351-362, 2012.

4. Zarghi H, Golian A, Kermanshahi H: The effect of triticale and enzyme cocktail (xylanase and β-glucanase) replacement in grower diet on performance, digestive organ relative weight, gut viscosity and gut morphology of broiler chickens [in Persian]. *Iranian J Anim Sci Res,* 2 (1): 298-312, 2016.

5. Algawany M, Elnesr SS, Farag MR: The role of exogenous enzymes in promoting growth and inproving nutrient digestibility in poultry. *Iranian J Vet Res,* 19 (3): 157-164, 2018. DOI: 10.22099/ijvr.2018.4932

6. Sarangi NR, Babu LK, Kumar A, Pradhan CR, Pati PK, Mishra P: Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on growth performance and carcass characteristics of broiler chickens. *Vet World*, 9 (3): 313-319, 2016. DOI: 10.14202/vetworld.2016.313-319

7. Yu B, Hsu JC, Chiou PWS: Effects of β-glucanase supplementation of barley diets on growth performance of broilers. *Anim Feed Sci Technol,* 70, 353-361, 1998. DOI: 10.1016/S0377-8401(97)00078-3

8. Jian-yi S, Wei-fen L, Zi-rong X, Sai-hong G: Purification and some properties of a β-glucanase from a strain trichoderma reesei GXC. *Zhejiang Univ Sci,* 3 (1): 106-112, 2002. DOI: 10.1007/BF02881853

9. Murakami AE, Franco JRG, Martins EN, Rondon O, Sakamato MI, Pereira MS: Effect of electrolyte balance in low-protein diets on broiler performance and tibial dyschonroplasia incidence. *J Appl Poult Res*, 12, 207-216, 2003. DOI: 10.1093/japr/12.2.207

10. Wang ZR, Qiao SY, Lu WQ, Li DF: Effect of enzyme supplementation on performance, nutrient digestibility gastrointestinal morphology and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. *Poult Sci*, 84, 875-881, 2005. DOI: 10.1093/ps/84.6.875

11. Milanković B, Ćirić J, Krstić M, Starčević M, Baltić B, Šefer D, Đorđević V, Popović M, Marković R: Effect of dietary fatty acid pattern on growth performance, carcass characteristics, fatty acid profile, and serum biochemistry parameters in broiler chickens. *Kafkas Univ Vet Fak Derg,* 25 (4): 507-516, 2019. DOI: 10.9775/kvfd.2018.21205

12. Poorghasemi M, Chamani M, Mirhosseini SZ, Sadeghi AA, Seidavi A: Effect of probiotic and different sources of fat on performance, carcass characteristics, intestinal morphology and ghrelin gene expression on broiler chickens. *Kafkas Univ Vet Fak Derg,* 24 (2): 169-178, 2017. DOI: 10.9775/kvfd.2017.18433

13. SPSS Inc: Statistical Package for Social Sciences Study. SPSS for Windows, Version 24. Chicago SPSS Inc., USA, 2016.

14. Agah MJ, Hashemi SMR, Noorolahi H, Hashemi M, Movasaghi S: Performance, carcass characteristics and some parameters of the gastrointestinal tract of broiler chickens fed with diets based on corn and triticale with or without the addition of enzyme supplement. *Anim Prod Res,* 7 (2): 35-46, 2018. DOI: 10.22124/ar.2018.7300.1201

15. Abudabos A: Enzyme supplementation of corn-soybean meal diets improves performance in broiler chickens. *Int J Poult Sci,* 9 (3): 292-297, 2010. DOI: 10.3923/ijps.2011.157.159

16. Mathlouthi N, Ballet N, Larbier M: Influence of beta-glucanase supplementation on growth performances and digestive organs weights of broiler chickens fed corn, wheat and barley-based diet. *Int J Poult Sci,* 10 (2): 157-159, 2011. DOI: 10.3923/ijps.2011.157.159

17. Kutlu HR, Saber SN, Kutay H, Celik L, Uzun Y, Toy N, Kutlu M, Yucelt O, Burgut A, Thiery P, Yavuz B: Effect of multi-enzyme produced by a single fungus on growth performance and some carcass parameters of broiler chicks fed on maize-soya based diets. *Kafkas Univ Vet Fak Derg,* 25 (2): 221- 230, 2019. DOI: 10.9775/kvfd.2018.20765

18. Zou J, Zheng P, Zhang K, Ding X, Bai S: Effects of exogenous enzymes and dietary energy on performance and digestive physiology of broilers. *J Anim Sci Biotechnol*, 4:14, 2013. DOI: 10.1186/2049-1891-4-14

19. Choct M, Annison G, Trimble RP: Soluble wheat pentosans exhibit different anti-nutritive activities in intact and cecectomized broiler chickens. *J Nutr*, 122, 2457-2465, 1992. DOI: 10.1093/jn/122.12.2457

20. Daymeh S, Afzali N, Bashtini M: Effect of revabio in diets containing wheat bran on growth performance, some blood metabolites and absorbing of mineral elements in broilers chickens. *Res Anim Prod*, 7, 33-43, 2016. DOI: 10.1016/j.carbpol.2005.11.033

21. Ajithkumar A, Andersson R, Siika-aho M, Tenkanen M, Aman P: Isolation of cellotriosyl blocks from barley B-glucan with endo-1,4-b-glucanase from *Trichoderma reesei*. *Carbohydr Polym,* 64, 233-238, 2006. DOI: 10.1016/j.carbpol.2005.11.033

22. Nahas J, Lefrancois MR: Effects of feeding locally grown whole barly with or without enzyme addition and whole wheat on broiler performance and carcass traits. *Poult Sci*, 80, 195-202, 2001. DOI: 10.1093/ps/80.2.195

23. Jenkis DJA, Axelsen M, Kendall CW, Augustin LSA, Vulsa V, Smith U: Dietary fibre, lente carbohydrates and the insulin-resistant diseases. *Br J Nutr*, 83, S157-S163, 2000. DOI: 10.1017/s0007114500001100

24. Smits CHM, Annison G: Nonstarch plant polysaccharides in broiler nutrition toward a physiologically valid approach to their determination. *World Poult Sci J*, 52, 203-221, 1996. DOI: 10.1079/WPS19960016

25. Basmacioğlu Malayoğlu H, Baysal Ş, Misirlioğlu Z, Polat M, Turan N, Yilmaz H: Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat-soybean meal diets. *Br Poult Sci,* 51 (1): 67-80, 2010. DOI: 10.5897/AJB12.1237

26. Khaksar V, Golian A, Kermanshahi H: Immune response and ileal microflora in broilers fed wheat-based diet with or without enzyme endofeed W and supplementation of thyme essential oil or probiotic primaLac. *Afr J Biotechnol,* 11 (81): 14716-14723, 2012.

27. Seidavi A, Dadashbeiki M, Alimohammadi-Saraei MH, van den Hoven R, Payan-Carreira R, Laudadio V, Tuffarelli V: Effects of dietary inclusion level of a mixture of probiotic cultures and enzymes on broiler chickens immunity response. *Environ Sci Pollut Res*, 24 (5): 4637-4644, 2017. DOI: 10.1007/s11356-016-8206-8

Kafkas Univ Vet Fak Derg
26 (2): 225-230, 2020
DOI: 10.9775/kvfd.2019.22777

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Genetic Characterization of *Gigantocotyle explanatum* from Buffaloes in Northwestern Pakistan

Sana ULLAH [1,a] Kiran AFSHAN [1,b1] Muhammad ARSHAD [1,c] Sabika FIRASAT [1,d]

[1] Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, PAKISTAN
[a] ORICD: 0000-0002-2450-8728; [b] ORCID: 0000-0002-3979-7606; [c] ORICD: 0000-0003-2720-4129; [d] ORCID: 0000-0003-4959-7832

Article ID: KVFD-2019-22777 Received: 31.05.2019 Accepted: 07.11.2019 Published Online: 08.11.2019

How to Cite This Article

Ullah S, Afshan K, Arshad M, Firasat S: Genetic characterization of *Gigantocotyle explanatum* from buffaloes in northwestern Pakistan. *Kafkas Univ Vet Fak Derg,* 26 (2): 225-230, 2020. DOI: 10.9775/kvfd.2019.22777

Abstract

The family Paramphistomidae including *Gigantocotyle explanatum* regularly infects ruminants and causes immense economic losses to the livestock industry by decreasing dairy products and growth rates. The present study was aimed to determine the novel molecular data of *G. explanatum* in Pakistan using ribosomal DNA (ITS1-5.8S, ITS2) regions. Adult flukes, *G. explanatum*, were collected from bile ducts of infected buffaloes. The most relevant sequences from the other parts of the world were downloaded from the GenBank. High intraspecific variations were obtained at 5 end region of ITS1 gene. The 3 end of ITS1 was conserved and showed 96% similarity with *Paramphistomum cervi* (KJ459936). The nucleotide blast search of 5.8S gene revealed that 40 sequences from trematodes had 98% to 99% identity with present sequence and found genetically identical to *P. cervi* (KJ459938) and *Dicrocoelium chinensis* (KF734784) from China. The ITS2 gene of investigated isolates showed no variation with Myanmar (AB743577), while blast search revealed 96-100% similarity with isolates from Myanmar, India, Bangladesh and China. This study demonstrates the utility of ITS2 and 3´ end ITS1 sequences as a valuable tool for elucidating species phylogenetic relationship in south Asia. This sequencing data will facilitate more accurate identification of *G. explanatum,* enabling future work to resolve many ambiguities in the literature regarding this species.

Keywords: *Gigantocotyle explanatum; Water buffaloes, Pakistan, Genetic characterization*

Kuzeybatı Pakistan'daki Buffalo'lardan *Gigantocotyle explanatum*'un Genetik Karakterizasyonu

Öz

Gigantocotyle explanatum'un da dahil olduğu Paramphistomidae familyası ruminantları sistemli olarak enfekte ederek süt üretimi ve büyüme oranlarını azaltır ve hayvancılık endüstrisinde büyük ekonomik kayıplara neden olur. Bu çalışmada Pakistan'da *G. explanatum*'un yeni moleküler verilerinin ribozomal DNA (ITS1-5.8S, ITS2) bölgeleri kullanılarak belirlenmesi amaçlandı. Yetişkin *G. explanatum* kurtçukları, enfekte buffaloların safra kanallarından toplandı. Dünyanın diğer bölgelerindeki en yakın gen dizileri GenBank'tan indirildi. ITS1 geninin 5'- uç bölgesinde yüksek intraspesifik varyasyonlar elde edildi. ITS1'in 3'- ucu korundu ve *Paramphistomum cervi* (KJ459936) ile %96 oranında benzerlik belirlendi. 5.8S geninin nükleotit BLAST araştırması, trematodlardan elde edilen 40 sekansın, mevcut sekans ile %98 ila %99 özdeşliğe sahip olduğunu ve genetik olarak Çin'den *P. cervi* (KJ459938) ve *Dicrocoelium chinensis* (KF734784) ile benzer olduğunu gösterdi. İncelenen izolatların ITS2 geni, Myanmar (AB743577) ile hiçbir farklılık göstermezken, BLAST araştırması, Myanmar, Hindistan, Bangladeş ve Çin'den elde edilen izolatlarla %96-100 oranında benzerlik gösterdiğini ortaya koydu. Bu çalışma, ITS2 ve ITS1 3'- uç dizilerinin Güney Asya'daki türlerin filogenetik ilişkisinin aydınlatılmasında değerli bir araç olduğunu göstermektedir. Bu veri dizini, *G. explanatum*'un daha doğru bir şekilde tanımlanmasını kolaylaştıracak ve bu türlerle ilgili literatürdeki birçok belirsizliğin çözülmesinde yeni araştırmalara olanak sağlayacaktır.

Anahtar sözcükler: *Gigantocotyle explanatum; Su bufalosu, Pakistan, Genetik karakterizasyon*

INTRODUCTION

Gastrointestinal parasitic infections that are caused by platyhelminths (digenean trematodes) of the family Paramphistomidae (Fishoeder, 1901), have been identified under 19 genera comprising more than 70 species [1]. *G. explanatum* causes great economic losses in terms of reduced growth rate, decline in milk and meat production and high morbidity rate of infected animals. In Pakistan its overall occurrence is still in infancy and various districts of Punjab have 17.39% to 44.44% prevalence [2].

İletişim (Correspondence)
+92 51 90643252
kafshan@qau.edu.pk

The morphologically similar trematodes show marked differences in epidemiological factors including pathogenicity, infectivity and response to anthelmintics. Thus molecular studies based on nuclear ribosomal DNA regions have proven to be of great importance in accurate identification of digenean parasite at different systematic levels [3]. Intraspecific variation is limited in rDNA spacer regions of animals and especially in helminth [4]. The ITS1 spacer regions contain a highly variable 5´ region which has been helpful in lower taxonomic studies while on the other hand their 3´end are more conserved that helps in high-level classifications. The sequence diversity of ITS1-5.8S rDNA has been studied in *Clonochis sinensis* in Russia [5] and *Paramphistomum cervi* in China [6]. The rDNA ITS2 is a very useful marker for correct identification of species and has been used for many Paramphistomatidae including *G. explanatum* [7] Fasciolidae [8], and Dicrocoeliidae [9]. The ITS1 and ITS2 markers are highly conserved regions, and can be used to differentiate the closely related taxa that have diverged very recently i.e. <50 million years ago [10].

Although, considerable attention has been paid to adverse pathology and epidemiology of *G. explanatum* in Pakistan, relatively little attention has been paid to the systematics of the species. So far, a single report on molecular characterization of *G. explanatum* comes out from Punjab [11]. Thus, the aim of this study was to determine the genetic structure of *G. explanatum* in Pakistan by using the complete ribosomal DNA (ITS1-5.8S-ITS2) regions. Such valuable information can be used for truthful understanding of the molecular mechanism, successful species adaptation, evolution and the maintenance of the ability of the parasite to infect.

MATERIAL and METHODS

Fluke Collection sites and Genomic DNA Extraction

A study was designed to determine the presence of *G. explanatum* species in liver and bile duct of buffaloes at various abattoirs of Peshawar Khyber Pakhtunkhwa (34.95° N 72.33° E). At least 8 visits per month were made to local buffalo slaughter houses at Peshawar from February 2018 to the last week of October 2018. Adult flukes were collected from the bile duct of infected buffaloes by forceps soon after their slaughtering. Individual liver flukes were washed several times in phosphate buffer solution (PBS) and identified by superficial morphological features [10]. Flukes of the same population were preserved in 70% ethanol and stored at -80ºC. Genomic DNA was extracted from 17 worms using the consecutive three-day protocol of Phenol Chloroform method described by Barker (1998) [12].

PCR Amplification and Sequence Analysis of ITSI-5.8S-ITS2

ITSI-5.8S gene was successfully amplified by polymerase chain reaction with universal primers: BD1F (5´GTCGT AACAAGGTTTCCGTA-3´) and BD2R (5´TATGCTTAAATTCA GCGGGT-3´); while another set of universal primers F=GGT GGATCACTCGGCTCGTG, R=TTCCTCCGCTTAGTGATATGC were used for the amplification of ITS2 gene. The PCR product had a total volume of 25 µL containing 2.5 µL dNTPs, 2 µL $MgCl_2$, 0.3 µL Taq polymerase, 0.5 µL each forward and reverse primer, 2.5 µL PCR buffer, 14.7 µL PCR water and 2 µL DNA. Thermo-cycler condition was maintained at 95ºC for 45 sec followed by 35 cycles at 95ºC for 45 sec, 61ºC for 45 sec and 72ºC for 90 sec with a final extension process at 72ºC for 10 min. PCR products were purified using Wizrep purification mini Kit (Wizbio solutions), and both DNA strands were sequenced through Macrogen Sequencing services (Korea). The rDNA ITSI-5.8S-ITS2 sequence was assembled, aligned and edited to remove the attached primer and extra poor sequences on both ends using Chromas software. Sequences relatively close to this species from other geographical regions were downloaded from NCBI GenBank. The ribosomal DNA sequences were aligned using CLUSTALW.

Phylogenetic Analysis of the rDNA ITSI-5.8S-ITS2

Once species variations for the rDNA sequences had been identified, the under study haplotypes were imported into MEGA 7 [13] to calculate the suitable model of nucleotide substitution to construct their phylogeny. The most suitable node was formulated by rooting the branches to their closely related species. The phylogenetic tree was selected by the maximum likelihood method. Primary tree(s) for the heuristic search were attained automatically by selecting Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances calculated by the maximum composite likelihood (MCL) method, and then selected the topology with superior log likelihood value.

RESULTS

Successfully sequenced *Gigantocotyle explanatum* (n=17) rDNA genes were checked for intraspecific variations. The sequence was edited to length 1089 bp *(Fig.1)* with a total length of identified ITSI is 650 bp, 5.8S 157 bp and ITS2 282 bp.

The total length of identified ITSI is 650 bp, and at 3´ end 400 bp were completely identical to each other with no intraspecific variation. All the sequences of ITS1 region of *G. explanatum were* trimmed to 400bp and aligned with 9 closely related available sequences using BLAST. ITSI sequences of current parasite showed maximum similarity with *Paramphistomum cervi* of China and varied with other digenean. The present results showed 98% similarity with *Paramphistomum cervi* of China (KJ459936) with 8 nucleotide variations at positions 40 (T>A), 44 (A>T), 110 (A>G), 149 (G>A), 180 (T>C), 181 (G>T), 211 (C>T) and 382 (A>G) respectively. A consistent sequence variation was observed with the two species of *Gastrodiscoides hominis*

Fig 1. Identified sequence of ITS1-5.8S-ITSII region of *G. explanatum* from infected buffaloes of Khyber Pakhtunkhwa Pakistan

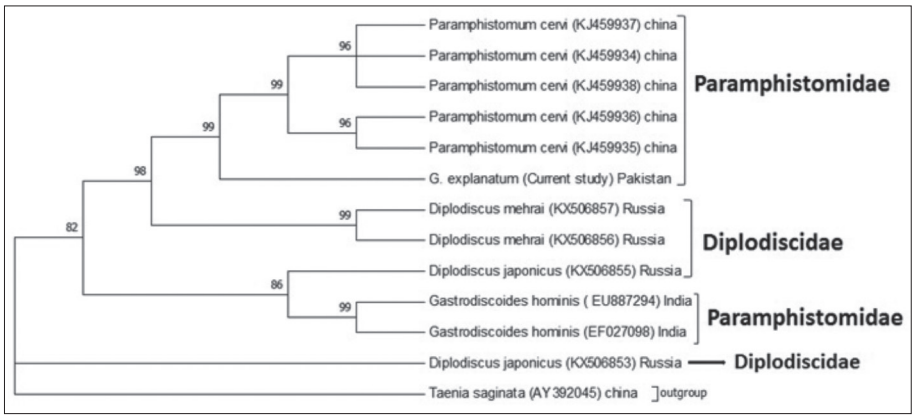


Fig 2. Molecular phylogenetic analysis of ITS-I gene of *G. explanatum* by maximum likelihood method

(EF027098), (EU887294) of 26bp at different positions from India but showed major differences with the same species of Columbia. The main types of mutations in the current study were A↔G and T↔C transition at 6 different positions and G↔A transition occured two times in the region with *Paramphistomum cervi* (KJ459934) of China. In addition, C↔T, A↔G, G↔A, T↔C, transition and A↔T, G↔T transversion mutation was found in various positions with *Watsonius watsoni* (KC763806) of the same country. Phylogenetic tree for ITSI revealed that the current species of *G. explanatum* formed a sub clade with *Paramphistomum cervi* (KJ459936) a rumen parasite of China with 99% similarity. Two species of *Gastrodiscoides hominis* (EU887294) from India also make a sub clade with 86% similarity index. Comparison with other paraphyletic clades as follows *Diplodiscus mehari* (KX506857) and *Diplodiscus japonicus* KX506855 of Russia with 82% bootstrap values *(Fig. 2)*. *Taenia saginata* (AY392045) of China considered as an out group.

Nucleotide blast search revealed that 40 sequences had 98% to 99% identity with the present sequence of 5.8S rDNA. The complete 5.8S region of the current species demonstrated no intra-specific variation and the Pakistani *G. explanatum* was genetically identical to the two species of *Paramphistomum cervi* (KJ459938) and *Dicrocoelium chinensis* (KF734784) from China. Interspecific single nucleotide polymorphisms (SNPs) have been identified between the sequences of genus *Centrocestus*, *Diplodiscus*, *Gynichthys, Acanthostomum, Chiorchis, Cryptocotyle, Siphomutabilus*. The *Apophallus* species from Hungary showed variation at positions 69,109,120 respectively. Nonetheless, genus *Homalometron* and *Thysanopharynx* sequence exhibit 3 fixed variable sites with present sequence. Russian *Clonorchis sinensis* showed variation at 4 positions with current species. Phylogenetic analysis for 5.8S in *Fig. 3* supports the blast results, by showing similarity with *Paramphistomum cervi* (KJ459938) of China with high bootstrap value (99%). The scrutiny involved 11 nucleotide sequences. All gaps and missing data were removed. The computed phylogram revealed 4 major clades. The *G. explanatum* formed a tight cluster with

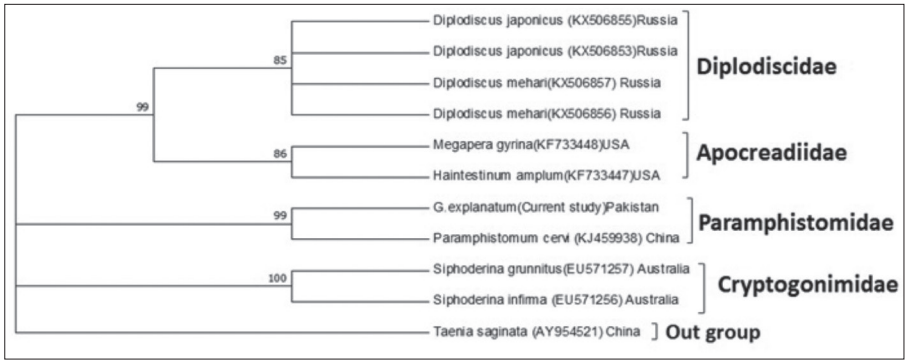
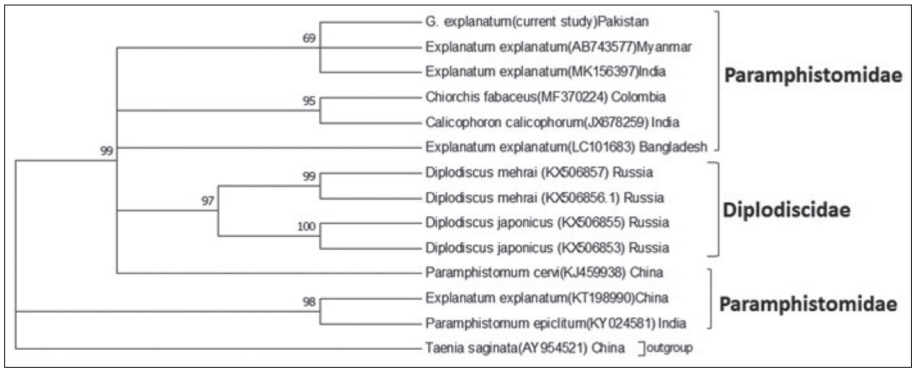


Fig 3. Molecular phylogenetic analysis of rDNA 5.8S gene of *G. explanatum* by maximum likelihood method

Fig 4. Molecular phylogeny based on rDNA ITS-2 sequence data of *G. explanatum*



family Paramphistomidae, Diplodiscidae, Apcreadiidae, and Cryptogonimidae, respectively. *Taenia saginata* (AY954521) from China showed a separate clade as out group.

The ITS2 sequences were checked for intraspecific variations and found identical. All the sequences were identified correctly and showed 96-100% similarity with reference sequences of *G. explanatum* from Myanmar, India, Bangladesh and China. The total sequence length was 282 bp and compared with the reference sequences for interspecific variations. The sequence was identical with Myanmar (AB743577) and showed difference of 1 bp (0.34%) at position of 253rd (C>A) with Bangladesh (LC101683), 1 bp (0.34%) difference with India (KF564869) at position 198 (C>T) and differ from China (KT198990) by 2 bp (0.69%) at 71st (T>C) and 218th (C>T) positions. However, the results differ from Indian isolates (KC503920, JX678250) by 7 bp (2.44%) and 13 bp (4.54%) respectively. The identified isolates within various taxa of Paramphistomidae showed variation from 10-16 bp. ITS2 tree indicated that identified isolates form a sub-clad with isolates of Paramphistomidae and Diplodiscidae, Gastrothylacidae families *(Fig. 4)*. However, the *Taenia saginata* (AY954521) diverged into separate clade as out group.

DISCUSSION

The current study sequenced the ITSI-5.8S-ITS2 of ribosomal DNA. The ITS2 sequences analysis confirmed the *G. explanatum* identity. The identification of this parasite has been previously confirmed in our neighboring countries i.e. Myanmar [8], Iran [14], India [15], Bangladesh and Nepal [16]. A study from Pakistan [11] identified the ITS2 region of *G. explanatum* as well from Rawalpindi, Punjab province.

The 5.8S showed 100% identity with number of trematodes species which indicates that this small region is highly conserved and not a reliable marker for species-level identification. However, ITS1 region of Paramphistomidae is usually characterized to infer the intraspecific variation among species [17]. Our results manifested high similarity index between *G. explanatum* of current study, and Chinese *Paramphistomum cervi* (KJ459936), and showed differences with each other at only 8 nucleotides. ITS1 regions of three opisthorchid liver fluke species *C. sinensis, O. viverrini, O. felineus* revealed high degree of interspecific sequence divergence and small amount of intraspecific variation in 42 individuals from eight different geographical localities that gives strong arguments that these species shared same ancestors and spread through the movement of infected host across different countries [18]. Zheng et al.[6] reported that no genetic difference is detected in 5.8S within species and only a small amount of intraspecific variation 0.04% is found in ITS-I region of *P. cervi* of China. This high level of similarity determined that these species are present in geographically linked countries and may share evolutionary history [19].

In addition, another digenean *Paragonimus westermani* showed valuable amount of intra individual differences as well as with other species of the same genus in amplified ITS1 region. The reason behind these variations is the varying number of repeat sequences in some species of digenean [20]. The homogenization of these repeat sequences of ribosomal

DNA is due to concerted evolution [21]. These repeat sequences are not operating uniformly in all genera of parasite species [20]. So this statement in turn supports the fact that no such type of successive repeats sequences was observed in our current ITS1region of *G. explanatum* from Pakistan. Different categories of ITS1 variation have been observed in various studies on trematodes DNA. This difference in degree of change in ribosomal DNA tandem array is probably due to the cluster segregation during replication that also enables to maintain homogeneity in their sequences [22].

The molecular analysis of ITS2 rDNA is very important for intraspecific and interspecific variation. The documented study showed that ITS-2 region of *G. explanatum* is highly conserved and hardly ever shows the intraspecific variation. But it differs interspecifically to other members of family i.e. from *Paramphistomum leydeni* at 7 nucleotide sites [8,11]. The current sequence ITS2 of 286 bp was genetically identical to *G. explanatum* of Myanmar (AB743577) and a difference of single nucleotide with Bangladesh (LC101683), and single nucleotide difference reported with Myanmar [11]. Interspecific variations were one to two nucleotides with India (KF564869) and China (KT198990) respectively. These minor differences of nucleotides may indicate that the haplotypes of these countries are closely related. The literature data shows that the interspecific variations of ITS2 of trematode are in range of 0.3 to 21%, which is very wide [9]. In contrary, it is believed that generally the ITS2 region shows very slow rate of evolution and in some cases demonstrate a complete absence of intraspecific and interspecific nucleotide variations [9,23]. But still, the ITS1 and ITS2 regions are the most frequently used markers for studying of population genetic and applications of evolutionary biology in digeneans parasites [24]. So the ITS2 is considered a highly significant genetic marker for the study of intraspecific as well as interspecific variation of species. Because the differences of even one nucleotide change can be used as an effective genetic marker for distinguishing the closely related species of digeneans [25].

The Phylogenetic tree is very useful in providing the hypothesis about the clade of various species and link between different species [26]. The Phylogenetic tree of our study showed that *G. explanatum* of our region forms clad with India, Myanmar, Bangladesh and China, confirming their closeness. Similarly, study reported that the haplotypes of *G. explanatum* found in India showed same clade with isolates from Bangladesh and Nepal [27]. Our systematic analysis of the ITSI and 5.8S revealed the closeness of current species of *G. explanatum* with *Paramphistomum cervi* (KJ459936) of China. Genetic pair wise distance between current species of *G. explanatum* also confirmed their close affiliation with *P. cervi*. These results may explain that as these countries are geographically similar with mostly identical culture, and have movement of the ruminant among these countries and might be shared through migration of animals [28].

Although the goal of the present work is to be stressed that morphological discrimination does not provide adequate information about amphistomes identity, however the molecular data is needed to justify their accurate taxonomic structure. In conclusion the appropriate identification of flukes will help to minimize anthelmintic resistance. The novel sequence data will help in formulation of early diagnostic tools, effective drugs and specific vaccinations against these amphistomes. Furthermore, detail genetic studies on mitochondrial (cytochrome c oxidase subunit 1) regions of *G. explanatum* are required to resolve many ambiguities in the literature regarding this species.

REFERENCES

1. Jones A, Jones A, Bray RA, Gibson DI: Superfamily Paramphistomoidea Fischoeder, 1901, *The Natural History Museum London, UK*, 2005.

2. Iqbal M, Muhammad A, Anjum A, Shahzad K, Ali M, Ali S: Epidemiology of *Gigantocotyle explanatum* in naturally infected buffaloes. *Veterinaria,* 1 (1):15-18, 2014.

3. Anh DN, Anh LT, Tuan LQ, Bac ND, Tien TV, Phuong VTB, Duong TT, Luc NK, Quang NB: Identification of *Fasciola* species isolates from Nghe An province, Vietnam, based on ITS1 Sequence of ribosomal DNA using a simple PCR-RFLP method. *J Parasitol Res,* 2018:2958026, 2018. DOI: 10.1155/2018/2958026

4. Králová-Hromadová I, Štefka J, Špakulová M, Orosová M, Bombarová M, Hanzelová V, Bazsalovicsová E, Scholzb T: Intra-individual internal transcribed spacer 1 (ITS1) and ITS2 ribosomal sequence variation linked with multiple rDNA loci: A case of triploid Atractolytocestus huronensis, the monozoic cestode of common carp. *Int J Parasitol,* 40 (2): 175-181, 2010. DOI: 10.1016/j.ijpara.2009.07.002

5. Tatonova YV, Chelomina GN, Besprosvannykh VV: Genetic diversity of nuclear ITS1–5.8 S–ITS2 rDNA sequence in *Clonorchis sinensis* Cobbold, 1875 (Trematoda: Opisthorchidae) from the Russian Far East. *Parasitol Int,* 61 (4): 664-674, 2012. DOI: 10.1016/j.parint.2012.07.005

6. Zheng X, Chang QC, Zhang Y, Tian SQ, Lou Y, Duan H, Guo DH, Wang CR, Zhu XQ: Characterization of the complete nuclear ribosomal DNA sequences of *Paramphistomum cervi*. *Sci World J,* 2014:751907, 2014. DOI: 10.1155/2014/751907

7. Itagaki T, Tsumagari N, Tsutsumi KI, Chinone S: Discrimination of three amphistome species by PCR-RFLP based on rDNA ITS2 markers. *J Vet Med Sci,* 65 (8): 931-933, 2003. DOI: 10.1292/jvms.65.931

8. Mas-Coma S, Valero MA, Bargues MD: Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol*, 69, 41-146, 2009. DOI: 10.1016/S0065-308X(09)69002-3

9. Gorjipoor S, Moazeni M, Sharifiyazdi H: Characterization of *Dicrocoelium dendriticum* haplotypes from sheep and cattle in Iran based on the internal transcribed spacer 2 (ITS-2) and NADH dehydrogenase gene (nad1). *J Helminthol*, 89 (2): 158-164, 2015. DOI: 10.1017/S0022149X13000679

10. Sey O: Keys to the Identification of the Taxa of the Amphistomes (Trematoda, Amphistomida), University of Pecs, Hungary, 2005.

11. Chaudhry U, van Paridon B, Lejeune M, Shabbir MZ, Rashid MI, Ashraf K, Ashraf S, Gilleard J, Sargison N: Morphological and molecular identification of *Explanatum explanatum* in domestic water buffalo in Pakistan. *Vet Parasitol Reg Stud Reports,* 8, 54-59, 2017. DOI: 10.1016/j.vprsr.2017.02.002

12. Barker DJ: In utero programming of chronic disease. *Clin Sci,* 95 (2): 115-128, 1998.

13. Kumar S, Stecher G, Tamura K: MEGA7 molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Evol,* 33 (7): 1870-

1874, 2016. DOI: 10.1093/molbev/msw054

14. Mazahery Y, Razmyar J, Hoghooghi-Rad N: *Explanatum explanatum* [Creplin, 1847] Fukui, 1929, in buffaloes in the Ahwaz area, southwest Iran. *Vet Parasitol,* 55 (1-2): 149-153, 1994. DOI: 10.1016/0304-4017(94)90066-3

15. Hayashi K, Mohanta UK, Ohari Y, Neeraja T, Singh TS, Sugiyama H, Itagaki T: Molecular characterization and phylogenetic analysis of *Explanatum explanatum* in India based on nucleotide sequences of ribosomal ITS2 and the mitochondrial gene nad1. *J Vet Med Sci,* 78 (11): 1745-1748, 2016. DOI: 10.1292/jvms.16-0252

16. Mohanta U, Rana H, Devkota B, Itagaki T: Molecular and phylogenetic analyses of the liver amphistome *Explanatum explanatum* (Creplin, 1847) Fukui, 1929 in ruminants from Bangladesh and Nepal based on nuclear ribosomal ITS2 and mitochondrial nad1 sequences. *J Helminthol,* 91 (4): 497-503, 2017. DOI: 10.1017/S0022149X16000420

17. Van Herwerden L, Blair D, Agatsuma T: Intra-and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. *Parasitology,* 116 (4): 311-317, 1998. DOI: 10.1017/S003118209800242X

18. Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, Fukuda K: Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology,* 131 (5): 679-685, 2005. DOI: 10.1017/S0031182005008292

19. Blair D, Campos A, Cummings M, Laclette J: Evolutionary biology of parasitic platyhelminths: the role of molecular phylogenetics. *Parasitol Today,* 12 (2): 66-71, 1996. DOI: 10.1016/0169-4758(96)80657-0

20. Tang J, Toe L, Back C, Unnasch TR: Intra-specific heterogeneity of the rDNA internal transcribed spacer in the *Simulium damnosum* (Diptera: Simuliidae) complex. *Mol Bio Evol*, 13 (1): 244-252, 1996. DOI: 10.1093/oxfordjournals.molbev.a025561

21. Dover G: Molecular drive: A cohesive mode of species evolution. *Nature*, 299, 111-117, 1982. DOI: 10.1038/299111a0

22. Kane RA, Ridgers IL, Johnston DA, Rollinson D: Repetitive sequences within the first internal transcribed spacer of ribosomal DNA in schistosomes contain a Chi-like site. *Mol Biochem Parasitol,* 75 (2): 265-269, 1996. DOI: 10.1016/0166-6851(95)02525-1

23. Biémont C, Vieira C: Junk DNA as an evolutionary force. *Nature,* 443, 521-524, 2006. DOI: 10.1038/443521a

24. Herrmann KK, Poulin R, Keeney DB, Blasco-Costa I: Genetic structure in a progenetic trematode: Signs of cryptic species with contrasting reproductive strategies. *Int J Parasitol,* 44 (11): 811-818, 2014. DOI: 10.1016/j.ijpara.2014.06.006

25. Hillis DM, Dixon MT: Ribosomal DNA: Molecular evolution and phylogenetic inference. *Q Rev Biol,* 66 (4), 411-453, 1991. DOI: 10.1086/417338

26. Shameem H, Devada K, Lakshmanan B, Joseph S, Sabu L, Usha AP: Morphological and molecular characterisation of common amphistome species from cattle of South India. *J Vet Med Allied Sci,* 2 (1): 7-11, 2018.

27. Laidemitt MR, Zawadzki ET, Brant SV, Mutuku MW, Mkoji GM, Loker ES: Loads of trematodes: Discovering hidden diversity of paramphistomoids in Kenyan ruminants. *Parasitology*, 144 (2): 131-147, 2017. DOI: 10.1017/S0031182016001827

28. Mohanta UK, Ichikawa-Seki M, Shoriki T, Katakura K, Itagaki T: Characteristics and molecular phylogeny of Fasciola flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. *Parasitol Res,* 113 (7): 2493-2501, 2014. DOI: 10.1007/s00436-014-3898-5

Kafkas Univ Vet Fak Derg
26 (2): 231-238, 2020
DOI: 10.9775/kvfd.2019.22784

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Evaluating the Contribution of Acid Resistance Systems and Probing the Different Roles of the Glutamate Decarboxylases of *Listeria monocytogenes* Under Acidic Conditions

Chun FANG [1,2,3,†,a] Xiaowei FANG [1,†,b] Xueyang CHEN [1,c] Chen WANG [1,d]
Xiongyan LIANG [1,e] Yufang GU [1,f] Weihuan FANG [2,g] Yuying YANG [1,h]

[†] *These authors contributed equally to this work*
[1] School of Animal Science, Yangtze University, No.88, Jingmi Road, Jingzhou, 434025, CHINA
[2] Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine and Institute of Preventive Veterinary Medicine, Zhejiang University, No.866, Yuhangtang Road, Hangzhou, 310058, CHINA
[3] Key Laboratory of Prevention and Control Agents for Animal Bacteriosis (Ministry of Agriculture), Institute of Animal Husbandry and Veterinary Sciences, Hubei Academy of Agricultural Sciences, No.29, Nanhu Road, Wuhan, 430064, CHINA
[a] ORCID: 0000-0002-4599-9060; [b] ORCID: 0000-0001-7402-7255; [c] ORCID: 0000-0001-9998-1811; [d] ORCID: 0000-0003-4099-3820
[e] ORCID: 0000-0002-6256-5600; [f] ORCID: 0000-0001-6109-4651; [g] ORCID: 0000-0002-2460-0743; [h] ORCID: 0000-0002-0957-8373

Article ID: KVFD-2019-22784 Received: 05.06.2019 Accepted: 09.10.2019 Published Online: 09.10.2019

How to Cite This Article

Fang C, Fang X, Chen X, Wang C, Liang X, Gu Y, Fang W, Yang Y: Evaluating the contribution of acid resistance systems and probing the different roles of the glutamate decarboxylases of *Listeria monocytogenes* under acidic conditions. *Kafkas Univ Vet Fak Derg,* 26 (2): 231-238, 2020. DOI: 10.9775/kvfd.2019.22784

Abstract

Listeria monocytogenes is an important zoonotic foodborne pathogen, which can cause a severe invasive illness to susceptible humans and animals with high mortality. As *L. monocytogenes* is widely distributed in natural environments, the bacterium is easy to contaminate food processing facilities and the products to be ingested by host. But during the transition from a saprophyte to intracellular pathogen, one of the biggest challenge *L. monocytogenes* encounters is the acid stress. To combat the acidic environments, the bacterium developed several acid resistance systems, including acid tolerance response (ATR), F0F1-ATPase, glutamate decarboxylase (GAD), arginine deiminase (ADI) and agmatine deiminase (AgDI). In this study, we comprehensively evaluated the contributions of different acid resistance systems and explored the different roles of the three GAD components under acidic conditions. We found that the GadD2 of GAD system made the largest contribution to the survival of *L. monocytogenes* in artificial gastric juice (AGJ) and acidic brain heart infusion (BHI), which was followed by the global stress regulator SigB, GadD3 of GAD system, AguA1 of AgDI system and ArcA of ADI system. Transcription analysis showed that the mRNA level of the three GADs were consistent with their contribution to acid resistance. Similar results were observed in the other three representative strains EGDe, Lm850658 and M7. We further obtained the purified GADs and their poly-antibodies to demonstrate that the contribution of the three GADs were determined by the protein levels in *L. monocytogenes*. Further studies are needed to focus on the regulation of different expression of the GAD system.

Keywords: *Listeria monocytogenes, Acid resistance, Glutamate decarboxylase, Survival*

Asidik Koşullar Altında *Listeria monocytogenes*'in Glutamat Dekarboksilazlarının Asit Direnç Sistemlerine Katkılarının Değerlendirilmesi ve Farklı Rollerinin Araştırılması

Öz

Listeria monocytogenes, duyarlı insan ve hayvanlarda yüksek ölüm oranı ile seyreden bulaşıcı hastalıklara neden olabilen, önemli bir gıda kaynaklı zoonotik patojendir. *L. monocytogenes* doğal ortamlarda yaygın olarak bulunduğundan, gıda işleme tesislerinin ve konakçı tarafından tüketilen ürünlerin bakteri ile kontaminasyonu kolaydır. Ancak bir saprofitten hücre içi patojene dönüşmesi sırasında, *L. monocytogenes*'in karşılaştığı en büyük güçlüklerden biri asit stresidir. Asidik ortamlarla savaşmak için, bakteri, asit tolerans yanıtı (ATR), F0F1-ATPase, glutamat dekarboksilaz (GAD), arginin deiminaz (ADI) ve agmatin deiminaz (AgDI) dahil olmak üzere çeşitli asit direnç sistemleri geliştirmiştir. Bu çalışmada, farklı asit direnç sistemlerinin katkıları kapsamlı bir şekilde değerlendirildi ve üç GAD bileşeninin asidik koşullar altında farklı rolleri araştırıldı. GAD sistemindeki GadD2'nin, *L. monocytogenes*'in yapay mide sıvısı (AGJ) ve asidik beyin kalp infüzyonunda (BHI) hayatta kalmasına en büyük katkıyı yaptığı ve bunu GAD sisteminden global stres regülatörü SigB, GadD3 ile AgDI sisteminden AguA1 ve ADI sisteminden ArcA'nın izlediği belirlendi. Transkripsiyon analizi, üç GAD'nin mRNA seviyesinin, asit direncine katkıları ile tutarlı olduğunu gösterdi. Benzer sonuçlar, diğer üç temsilci suş olan EGDe, Lm850658 ve M7'de de gözlendi. Ayrıca, üç GAD'nin katkısının, *L. monocytogenes*'teki protein seviyeleri tarafından belirlendiğini göstermek için saflaştırılmış GAD'ler ve bunların poli-antikorlarını elde ettik. GAD sisteminin farklı ekspresyonlarının düzenlenme mekanizmasının anlaşılabilmesi için daha fazla çalışmaya ihtiyaç vardır.

Anahtar sözcükler: *Listeria monocytogenes, Asit direnci, Glutamat dekarboksilaz, Sağkalım*

İletişim (Correspondence)
+86 716 8060308
yangyycn@yangtzeu.edu.cn

INTRODUCTION

Listeria monocytogenes is a facultative anaerobic, gram-positive bacterium that is ubiquitous in natural environment as a saprophyte. In field environment, *L. monocytogenes* is thought to live off decaying plant material. Following ingestion by susceptible humans or animals, *L. monocytogenes* is capable of making the transition into a pathogen [1]. As an important zoonotic foodborne pathogen, *L. monocytogenes* could cause a severe invasive illness with high mortality in immunocompromised individuals [2,3]. Food-borne infection is the most common pathway of both epidemic and sporadic listeriosis, with 99% of human cases caused by consumption of contaminated food products [4]. Upon entering into the host gastrointestinal tract, *L. monocytogenes* adhere and invade various types of cells, including the phagocytic cells by the internalins InlA and InlB as well as Lap and InlP [5]. Following theentry into cell, *L. monocytogenes* must escape from host cell vacuoles via the pore-forming cytolysin listeriolysin O (LLO) and two phospholipases PI-PLC and PC-PLC quickly [6]. If not, the bacteria can be killed by the acidic endosome and digested by enzymes from the fused lysosome (phagolysosome). Entry into the cytosol, *L. monocytogenes* uses cytosolic nutrient to proliferate, then the bacteria spread to the neighboring cells by usurping actin polymerization as motile force by the bacterial surface protein ActA and with the help of InlC to relieve the cortical tension [7]. Then *L. monocytogenes* need to escape from the double membrane vacuoles to finish cell-to-cell spread with the help of LLO, PC-PLC and PI-PLC once again [5]. So it is not difficult to find that during the infection process, one of the biggest challenge *L. monocytogenes* encounters is acid stresses from stomach and phagolysosomes [8].

Listeria monocytogenes contains several enzyme systems including F_0F_1-ATPase, ADI, AgDI, GAD and acid tolerance response, to maintain intracellular pH homeostasis in acidic environments [9]. Under acid stress, F_0F_1-ATPase system uses ATP hydrolysis to produce proton motive force to pump cytoplasmic protons, while the ADI and AgDI use arginine and agmatine to produce ammonia to neutralize the cytoplasmic protons, respectively [10,11]. The glutamate decarboxylase (GAD) system, which consumes intracellular protons by converting glutamate to γ-aminobutyrate [12], also plays a role in acid resistance of *L. monocytogenes* to protect them in low pH foods. Moreover, pre-exposure of *L. monocytogenes* to mild acid could induce acid tolerance response (ATR) that improves the survival rate under fatal acid stress. As a global transcriptional regulator, SigB has been reported to positively regulate the ATR to help *L. monocytogenes* to deal with acid stress [13].

Although all the acid resistance systems were individually demonstrated to play important roles in acid stress and pathogenicity of *L. monocytogenes*, to date, no comprehensive assessment was conducted on these acid resistance systems, and the relative roles of these systems remain unclear. Moreover, *L. monocytogenes* contains several copies for some acid resistance systems. For example, *L. monocytogenes* 10403S has two AgDI genes *(aguA1* and *aguA2)*, and both of them were upregulated in response to acid stress, but only AguA1 contributed to acid resistance and pathogenicity of the bacteria [11]. For the GAD system, most of the *L. monocytogenes* strains (lineages I and II) contain three GADs [14], but the contributions of different GADs remain unclear. In this study, we tried to evaluate the contributions of different acid resistance systems and to clarify the different roles of the three GAD components under the acidic condition.

MATERIAL and METHODS

Bacterial Strains, Plasmids and Culture Conditions

Listeria monocytogenes 10403S, EGDe, Lm850658 and M7 were used as the wild-type strains. *Escherichia coli* DH5α was employed as the host strain for plasmids pET30a and pKSV7. *E. coli* Rosetta was used as expression host. *L. monocytogenes* and *E. coli* were cultured in brain heart infusion (BHI, Oxoid, Basingstoke, U.K.) and Luria-Bertani medium (LB, Oxoid), respectively, at 37°C. Stock solutions of ampicillin (50 mg/mL), kanamycin (50 mg/mL) and chloramphenicol (10 mg/mL; Sangong Biotech Co., Ltd, Shanghai, China) were added to the media, when appropriate, at the required concentrations.

Survival in AGJ or Acidic BHI Broth

Survival assay in artificial gastric juice (AGJ) or acidic BHI broth was conducted as in previous research [15]. *L. monocytogenes* wild-type and mutant strains were grown overnight at 37°C in BHI broth at pH 7.0 with shaking. The cultures were collected by centrifugation at 3000 g at 4°C for 10 min, washed and resuspended in phosphate buffered saline (PBS, 10 mM, pH 7.4) with the $OD_{600\ nm}$ adjusted to 1.0. Then 50 μL bacterial suspension was mixed in 950 μL AGJ (8.3 g proteose peptone, 3.5 g D-glucose, 2.05 g NaCl, 0.6 g KH_2PO_4, 0.11 g $CaCl_2$, 0.37 g KCl, 0.05 g bile salt, 0.1 g lysozyme and 13.3 mg pepsin dissolved in 1 liter distilled water with pH adjusted to 2.5 with HCl, filter sterilized) or BHI broth with indicated pH values (filter sterilized). After 1 h of incubation at 37°C, the mixtures were serially diluted and plated on BHI agar plates. The plates were incubated at 37°C for 24 h and viable bacteria were counted. Survival rate was calculated as percentage of survived bacteria after incubating in the acidic conditions for 1 h relative to the incubated bacteria. Data was reported as the mean ± SD of three independent experiments, each performed in triplicate.

Transcriptional Analysis

Overnight cultures of *L. monocytogenes* strains were inoculated into fresh BHI broth and grown to exponential phase ($OD_{600\ nm}$=0.25) or stationary phase ($OD_{600\ nm}$=0.6) at

37°C. One milliliter of each culture was treated with pH 4.5 BHI for an hour and then pelleted by centrifugation at 4°C. Total RNA was extracted using the Trizol reagent according to the manufacturer's instruction (Sangong Biotech Co., Ltd) and cDNA was synthesized with reverse transcriptase (TOYOBO Biotech Co., Ltd, Shanghai, China). Quantitative real-time PCR (qRT-PCR) was performed in 20 µL reaction mixtures containing SYBR green qPCR mix (TOYOBO (SHANGHAI) Biotech Co., Ltd) to detect the transcriptional levels of indicated genes on the iCycler iQ5 real-time PCR system (Bio-Rad, Hercules, California, U.S.A.) with specific primer pairs listed in *Table 1*. The housekeeping gene *gyrB* was selected as an internal control for normalization as previous research [16].

Construction of Deletion and Complementation Mutants

A homologous recombination strategy was used to construct the deletion mutants of *L. monocytogenes* 10403S according to the previous research [17] using the primer pairs listed in *Table 2*. The homologous fragments of overlapping PCR were purified and ligated to pMD18-T (TaKaRa, Beijing). After sequencing, the inserted fragments were digested with the indicated restriction enzymes, ligated to the temperature-sensitive shuttle vector pKSV7 and transformed into DH5α. Plasmids containing the inserted fragments were subsequently extracted and electroporated into *L. monocytogenes* competent cells. Transformants were grown at a non-permissive temperature (41°C) on BHI agar containing chloramphenicol (10 µg/mL) to promote chromosomal integration. The recombinants were passed in succession in BHI without antibiotic at a permissive temperature (30°C) to enable plasmid excision and curing. The deletion mutants were identified by PCR and confirmed by sequencing.

For the complementation strains, the encoding sequences of *gadD1*, *gadD2* and *gadD3* were amplified from *L. monocytogenes* EGDe with the indicated primer pairs listed in *Table 2*. After restriction digestion with appropriate enzymes, the PCR fragment was cloned into pIMK2 following the P_{Help} promoter. The recombinant plasmids were then electroporated into *L. monocytogenes* EGDe competent cells. The transformants were plated on BHI agar containing kanamycin (50 µg/mL) and positive clones were picked up and identified by PCR.

Prokaryotic Expression and Purification of GadD1, GadD2 and GadD3

GadD1, GadD2 and GadD3 were expressed as fusion proteins with His-tag using the expression vector pET30a (Invitrogen, U.S.A.) as previously shown [18]. The full-length *gadD1*, *gadD2* and *gadD3* were amplified with primer pairs listed in *Table 3*. The amplified fragments were cloned into

Table 1. *Primers used for q-PCR*

Primers	Sequences (5′-3′)	Size (bp)
gadD1-fwd	AGAATATCCACAGACAGCAAAG	142
gadD1-rev	CATAGCCATTCCACCAAGCAT	
gadT1-fwd	CGTTCTCGGTATTACAATTCCT	150
gadT1-rev	GCAAGCATGAAGATAACAAGAG	
gadT2-fwd	CCCTGTACCACTTATTATGGTT	116
gadT2-rev	CTACAGTTAAGGAAATTGCGGT	
gadD2-fwd	CCTTTGGAAAGATGAAAGCTAC	128
gadD2-rev	TGTAGTATTGACCGATGATGTG	
gadD3-fwd	ACCAATAATTTGGCTCGCACTA	144
gadD3-rev	TTAGTTTATCCGGGTGTTGGTT	
gyrB-fwd	AGACGCTATTGATGCCGATGA	91
gyrB-rev	GTATTGCGCGTTGTCTTCGA	

Table 2. *Primers used for deletion and complement mutants construction*

Primers	Sequences (5′-3′)	Size(bp)
gadD1-a	AATAAGCTTACTACACAGGTTTACAAGCA	515
gadD1-b	ACTCTCCCATTTTTCATAAATTCCTCCA	
gadD1-c	GAAAAATGGGAGAGTGATAAAATTTCTAG	524
gadD1-d	GCTGAATTCTTTTAATTGAAGTAACGTCA	
gadD1-e	AACCAACAGAAACATCGCTTCGTAT	
gadD2-a	ATAGCATGCCACTTATTATGGTTCAAG	536
gadD2-b	GATTTTTTCCTCCTATAATTTGTCTTGATT	
gadD2-c	TAGGAGGAAAAAATCTTCACACATTAA	545
gadD2-d	ATAGAATTCGGACTTATTCCGAGTAATG	
gadD2-e	GCAGCACTTTGTTACTTTTTGAAGAAG	
gadD3-a	GCAGGATCCAGCTTCTACTCTAACATGGTTCACG	567
gadD3-b	TTATAGTGAAGACGACAAGCGAACTTGGATGGT GAGTCCGA	
gadD3-c	TTCGCTTGTCGTCTTCACTATAAAGC	605
gadD3-d	AACGGTACCCGAGCGTGTCTATCTCACTATTCAT	
gadD3-e	GAAATTGTCGATTCCGGTGATGACT	
gadD1-CF	CGGGATCCTATGTTTAAAACAAATGTTGAACAAA	1406
gadD1-CR	GGGGTACCTTAATGAGTAAAGCCATGTGT	
gadD2-CF	CGGGATCCCATGTTATATAGTAAAGAAAATAA	1412
gadD2-CR	GGGGTACCTTAATGTGTGAAGCCGTGGA	
gadD3-CF	CGGGATCCGATGCTTTATAGTGAAGACGACA	1421
gadD3-CR	GGGGTACCTTAGTGCGTAAATCCGTATGAA	

Sequences with underline were restriction enzyme sites

Table 3. *Primers used for expression of the glutamate decarboxylases*

Primers	Sequences (5′-3′)	Size (bp)
gadD1-exp-fwd	GGAGGTACCATGTTTAAAACAAATGTTGAACAAA	1407
gadD1-exp -rev	CCAGGATCCTTAATGAGTAAAGCCATGTGT	
gadD2-exp-fwd	GAAGGTACCATGTTATATAGTAAAGAAAATAAAGA	1413
gadD2-exp -rev	GCCGGATCCTTAATGTGTGAAGCCGTG	
gadD3-exp-fwd	GGAGGTACCATGCTTTATAGTGAAGACGACA	1422
gadD3-exp -rev	TCTGGATCCTTAGTGCGTAAATCCGTATGAA	

Sequences with underline were restriction enzyme sites

the pET30a after restriction digestion. The recombinant plasmids were transformed to expression host *E. coli* Rosetta competent cells. Positive clones were confirmed by sequencing and then grown in 200 mL of LB medium supplemented with 50 μg/mL kanamycin at 37°C until $OD_{600\ nm}$ of the cultures reached 0.6-0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added into the medium at a final concentration of 0.4 mM to induce expression of GadD1, GadD2 and GadD3 for 12 h at 15°C. Then IPTG-induced cell pellets were collected, resuspended in 50 mM PBS (pH 7.4), and disrupted with 100 cycles of sonication at 300W for 5 sec with intermittent cooling on ice for 10 sec (25 min in total). After centrifugation at 12.000 g for 20 min, the supernatant samples were collected and loaded onto a 2-mL prepacked nickel-chelated agarose gel column (Weishi-Bohui Chromtotech Co., Ltd, Beijing, China). The columns were washed with 50 mM PBS containing 500 mM NaCl and 30 mM imidazole, and the bound proteins were eluted with a linear gradient of 25-500 mM imidazole prepared in the same buffer. Expression and purification of the recombinant proteins were analyzed on a 10% SDS-PAGE gel followed by Coomassie Brilliant Blue staining.

Polyclonal Antibodies Preparation

The purified recombinant protein was used for raising polyclonal antibodies in New Zealand white rabbits according to the previous study [19]. Rabbits were first immunized with 500 μg protein emulsified by the equal volume of Freund's complete adjuvant (Sigma, St. Louis, U.S.A.) through subcutaneous injection. After two weeks, the rabbit was boosted subcutaneously three times with 250 μg protein emulsified by incomplete Freund's adjuvant (Sigma, St. Louis, U.S.A.) at two-week intervals. Rabbits were bled 10 days after the last immunization and sera were isolated from the whole blood to collect polyclonal antibodies. Animal experiments were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

Western Blot Analysis

Listeria monocytogenes wild type and mutant strains were cultured with BHI broth to stationary phase at 37°C, then treated with pH 4.5 BHI broth for an hour. Then bacteria were harvested by centrifugation and pellets were lysed with lysis buffer and then homogenized with a refiner. The supernatant of cell lysis was isolated by centrifugation and analyzed by 10% SDS-PAGE. GadD1, GadD2 and GadD3 were blotted and probed with respective polyclonal antibodies produced in this study. Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as loading control. Then HRP conjugated goat-anti-rabbit IgG (Sangong Biotech Co., Ltd) was used as the second antibody to probe GadD1, GadD2, GadD3 and GAPDH. The abundance of indicated proteins was evaluated with software Quantity One (Version 4.6.6, Bio-Rad, U.S.A.) to calculate the gray level of specific bands.

Statistical Analysis

All data comparisons were analyzed using the two-tailed homoscedastic Student's T-test. In all cases, differences with $P<0.05$ were considered as statistically significant. The GraphPad Prism 5 (Version 5, GraphPad, U.S.A.) software was used to produce the graphs.

RESULTS

To evaluate the contribution of different acid resistance systems of *L. monocytogenes*, we knocked out the acid resistance-associated enzymes genes in the background of the reference strain 10403S and then compared the survival rate of the mutants with the reference strain. Our data showed that mutant strain ΔgadD2 exhibited the lowest survival rate (0.005%) in AGJ, which was followed by strains Δ*sigB*, ΔgadD3, ΔaguA1, ΔarcA, ΔgadD1 and ΔaguA2 *(Fig. 1A)*. Among these mutants, only Δ*aguA2* and Δ*gadD1* did not show a significant difference on the survival rate of *L. monocytogenes* in the acidic condition. A similar result was also observed in pH 2.5 acidic BHI broth *(Fig. 1B)*. These data indicated that the contribution of the acid resistance-associated enzymes was quite different. In *L. monocytogenes* 10403S, GadD2 made the largest contribution to acid resistance, which was followed by SigB, GadD3, AguA1 and ArcA.

To elucidate the different roles of the components of GAD system in acid resistance, we analyzed the transcriptional

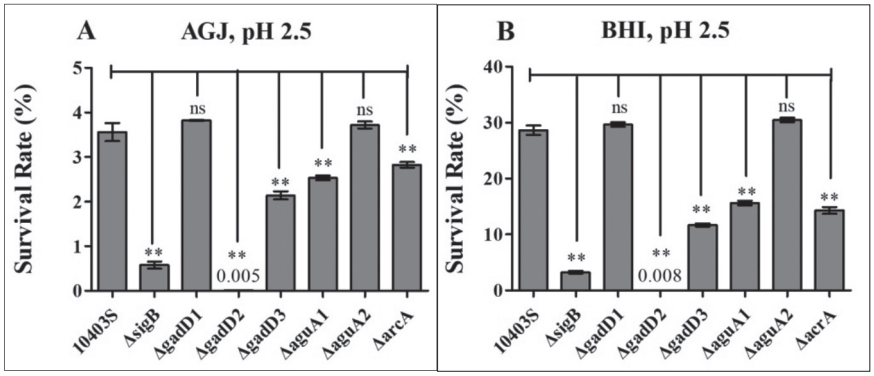


Fig 1. Survival of *L. monocytogenes* wild type 10403S and different mutant strains for an hour in pH 2.5 artificial gastric juice (AGJ) (A) or pH 2.5 brain heart infusion (BHI) broth (B). Experiments were conducted at least three times and values were expressed as mean ± SD. ** and ns indicate a statistically significant difference ($P<0.01$) and no significant difference between indicated strains, respectively

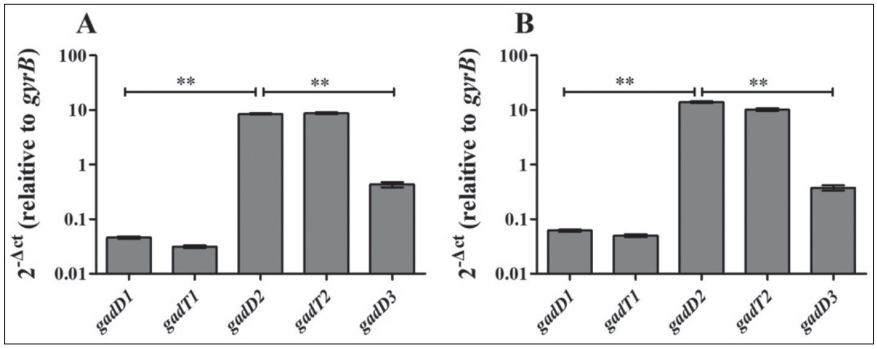


Fig 2. Relative transcriptional level of GAD system of *L. monocytogenes* wild type 10403S treated with pH 4.5 BHI for an hour at exponential phase (A) and stationary phase (B). Values were expressed as mean ± SD and ** indicate a statistically significant difference (P<0.01) between indicated strains

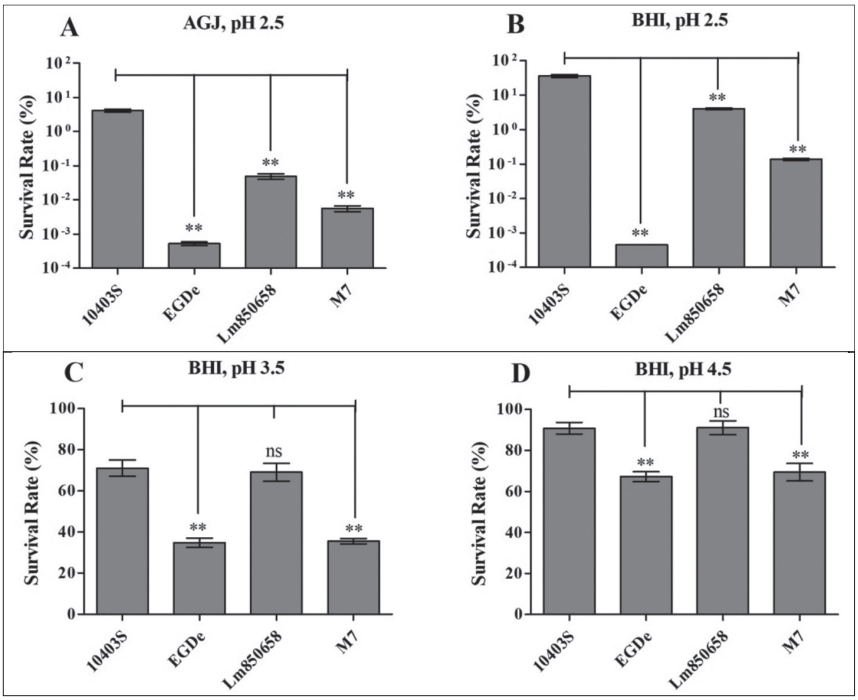


Fig 3. Survival of *L. monocytogenes* strains under different acidic conditions. Bacteria were treated with artificial gastric juice (AGJ) (A) or brain heart infusion (BHI) with pH 2.5 (B), pH 3.5 (C) and pH 4.5 (D) for an hour respectively. Experiments were repeated three times and values were expressed as mean ± SD. ** and ns indicate a statistically significant difference (P<0.01) and no significant difference between indicated strains, respectively

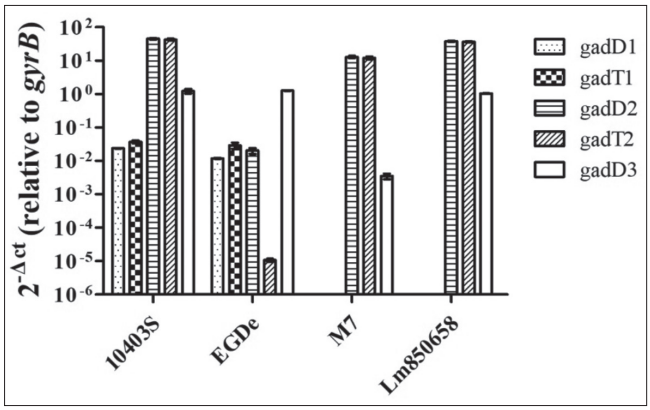


Fig 4. Relative transcriptional level of GAD system of the four *L. monocytogenes* wild type strains treated with pH 4.5 BHI for an hour at the stationary phase

level of the GAD system under acidic conditions. Our data showed that the transcriptional level of *gadT2/gadD2* was significantly higher than that of *gadD3* and *gadD1/gadT1* both at exponential and stationary phases in pH 4.5 BHI broth *(Fig. 2A,B)*. Moreover, the mRNA level of *gadD3* was ten-fold more than that of *gadD1/gadT1*. These results suggest that the contribution to acid resistance of different GAD components was correlative to their transcriptional level.

To confirm this hypothesis, we analyzed the survival rate of four representative strains, including 10403S, EGDe, Lm850658 and M7, under acidic conditions and the transcriptional level of their GAD system. Our data showed that strain 10403S exhibited the highest survival rate in AGJ for 1 h, which was followed by Lm850658, M7 and EGDe *(Fig. 3A)*. A similar result was also observed in pH 2.5 BHI broth *(Fig. 3B)*. When the pH increased to 3.5 or pH 4.5 in BHI, the survival rate of the four strains were increased, and the survival rate of 10403S and Lm850658 remained significantly higher than that of M7 and EGDe *(Fig. 3C,D)*. This survival rate was consistent with the transcriptional level, as the transcriptional level of *gadT2/gadD2* in EGDe and M7 were significantly lower than that of 10403S and Lm850658 in pH 4.5 BHI *(Fig. 4)*. The *gadD1/gadT1* mRNA level of EGDe was equal to that of 10403S, and *gadD1/gadT1* genes were deficient in lineage III strains

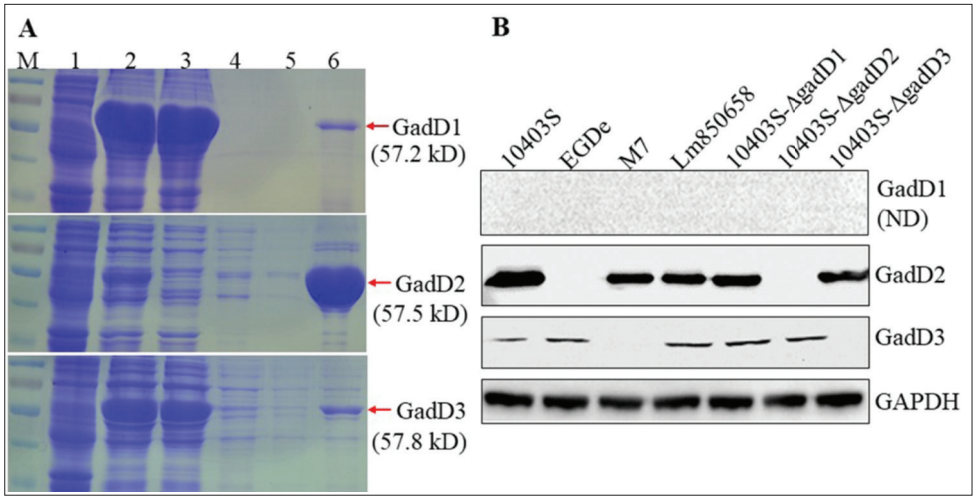
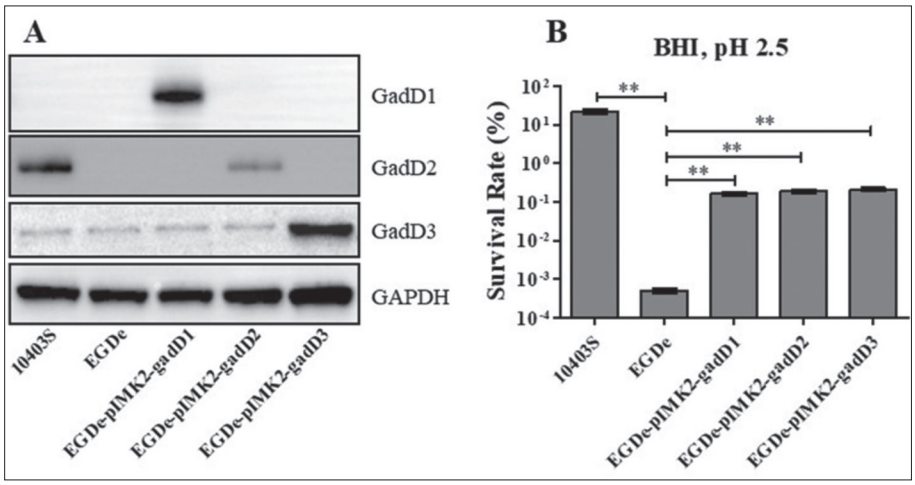


Fig 5. Affinity purification of GadD1, GadD2 and GadD3 (A) and expression of GadD1, GadD2 and GadD3 in the four wild type strains and the three mutant strains (B). M, Pre-stained Marker; lane 1, Control; lane 2 and 3, post-column sample; lane 4 and 5, wash with 4 mL 50 mM imidazole; lane 6, elution with 1 mL 400 mM imidazole; ND, not detected

Fig 6. Complementation of the GadD components rescued the survival of *L. monocytogenes* EGDe under acidic stress. Overexpression of GadDs (A) and survival of the indicated *L. monocytogenes* strains in pH 2.5 BHI broth for an hour (B). Values were expressed as mean ± SD and ** indicate a statistically significant difference (P<0.01) between indicated strains



Lm850658 and M7. The mRNA level of *gadD3* of 10403S, EGDe and Lm850658 were at the same level in the acidic condition, which was significantly higher than that of M7 *(Fig. 4)*.

To confirm whether the role of GAD system was determined by their expression, we purified prokaryotic expressed GadD1, GadD2 and GadD3 *(Fig. 5A)*. Poly-antibodies were obtained from immunized rabbit with the indicated purified proteins. Then the protein level of the three components in the acidic condition was detected by Western blot. The results showed that GadD1 was detected neither in wild type strain nor in the mutants *(Fig. 5B)*. GadD2 was not detected in *gadD2* mutant and EGDe, but highly expressed in 10403S and Lm850658 *(Fig. 5B)*. GadD3 was not detected in *gadD3* mutant and M7 *(Fig. 5B)*, which was similar to the tendency of its mRNA level *(Fig. 4)*. To confirm the hypothesis that the role of GAD in acid resistance was determined by their expression level, we overexpressed GadD1, GadD2 and GadD3 in EGDe to determine their function in acid resistance individually. Survival assay in pH 2.5 BHI broth showed that the overexpression any of the three GADs significantly improves the acid resistance of EGDe *(Fig. 6)*.

DISCUSSION

Listeria monocytogenes is a bacterium that lives in natural environments as a saprophyte but is capable of making the transition into a pathogen following its ingestion by susceptible humans or animals [1]. As an important zoonotic foodborne pathogen, this bacterium has the ability to adapt to a variety of environmental conditions [20]. Acidic environments such as silage, fermented foods, stomach and phagolysosomes, are the most common conditions that the bacterium encounters. *L. monocytogenes* contains several enzyme systems including F_0F_1-ATPase, ADI, AgDI and GAD to cope with these unfavorable conditions. Although all of the acid resistance systems had been demonstrated to play important roles in acid resistance in *L. monocytogenes* [9,21-24], it's unclear which system plays the major role in acid resistance of *L. monocytogenes*. Here we evaluated the effects of these systems on the survival of *L. monocytogenes* under acidic conditions. Our data showed that GadD2 of GAD system made the largest contribution to *L. monocytogenes* 10403S survival in different acidic conditions, which was followed by SigB, GadD3, AguA1, and ArcA. Since the constitution of these systems was complicated, it is difficult to knock out the whole system to

determine its contribution. In this study, we only knocked out the key enzymes of the acid resistance systems, which might be inadequate to evaluate the whole function of an acid resistance system, but we knocked out the whole AgDI system encoding region *(lmo0036-lmo0042)* at once. No significant difference was observed about the survival rate of strains Δ*aguA1(Δlmo0038)* and ΔAgDI *(Δlmo0036-lmo0042)* in acidic broth (data not shown). F_0F_1-ATPase system is essential for *L. monocytogenes*, in which mutantion will cause a lethal effect to the bacteria [25]. we didn't evaluate its contribution to acid resistance of *L. monocytogenes*. Datta et al.[25] and Cotter et al.[26] treated *L. monocytogenes* LS2 and LO28 at the exponential phase with N, N'-Dicyclohexylcarbodiimide (DCCD), an F_0F_1-ATPase inhibitor, which resulted in significantly reducing survival rate of the bacteria under the lethally acidic condition [25,26].

To better deal with acid stress, *L. monocytogenes* might employ several systems simultaneously. Moreover, the AgDI and GAD systems encode multiple isoenzymes, as two AgDI *(aguA1* and *aguA2)* and three GADs *(gadD1*, *gadD2* and *gadD3*) genes are encoded in most of *L. monocytogenes* [14,27]. The roles of these isoenzymes might make different contributions to the acid resistance of *L. monocytogenes*. As our previous study showed that *aguA1* and *aguA2* were both significantly up-regulated in response to acid conditions, only AguA1 but not AguA2 contributed to survival and growth under acidic environments and was involved in the pathogenicity of *L. monocytogenes* 10403S, in which Glycine 157 determined the activity of AguA1 and AguA2 [11]. In this study we found that the three GADs in *L. monocytogenes* 10403S also made different contribution (GadD2 > GadD3 > GadD1) to the survival of bacteria in acid stress. We further demonstrated that the function of the three GADs were determined by the expression level instead of their enzyme activity, which were different from AugA1 and AguA2 [11]. As our results showed that *L. monocytogenes* EGDe with little GadD1 and GadD2 was quite sensitive to acid stress, while overexpressed GadD1, GadD2 or GadD3 in EGDe with the *pHelp* promoter of plasmid pIMK2 significantly improved its survival rate in pH 2.5 BHI broth *(Fig. 3, 4, 6)*. Previous studies showed the difference of GAD system in *L. monocytogenes* and divided them into two groups, the outside GAD system (GAD_o, including GadD1/GadT1 and GadT2/GadD2) and the inside GAD system (GAD_i, GadD3) [23], and GAD_o played the major role in LO28 and 10403S, while acid resistance of EGDe was dependent on GAD_i [22,28]. These studies found the different roles of GAD system in various strains, but did not clarified the determinants that involved in the contribution in acid resistance of the GAD components. In this study, we demonstrated that the contribution of the three GADs was determined by their expression level for the first time.

The pH values and substrates that could be used by the bacterium to combat with low pH were various in different conditions [29], which might promote the bacterium to choose suitable acid resistance systems to cope with specific acidic environment. Whether these acid resistance systems perform the same in different conditions need further investigate. For instance, acid resistance in the phagosome might be complicated. On one hand, the bacteria need to initiate acid resistance system to cope with the acidic phagosome, on the other hand, the activation of LLO, which mediate *L. monocytogenes* escape from phagosome, need acidic compartment [30]. But food products contain glutamates that tend to benefit for the GAD system to deploy acid resistance [8]. Moreover, the molecular mechanisms involved in the different expression remained unclear. Kazmierczak et al.[31] found that *gadD3* was positivly regulated by SigB, and Cotter et al.[32] also found that *gadD1*/*gadT1* partially regulated by SigB. Bowman et al.[33] found that *gadT2*/*gadD2* operon was constitutively expression in *L. monocytogenes* LO28 by proteomic analysis. We found that the expression of *gadT2*/*gadD2* in the four representative strains were not in response to the acidic treatment, but the sequence of this operon (including the promoter region) was quite conserved between 10403S and EGDe or between Lm850658 and M7 (data not shown). It is suggested that different expression of *gadT2*/*gadD2* in these strains might be regulated in an undiscovered manner. Taken together, we demonstrated that the different contribution to acid resistance of GAD components were determined by the expression levels. The mechanisms that mediate the expression difference of GAD system need further investigation.

Statement of Author Contributions

CF and XWF designed and conducted experiments, analyzed data, and they were contributed equally to the work. CF, XWF, XYC, XYL, CW, YFG, WHF and YYY were involved in study design and data collection. YYY supervised the study and critically read the manuscript.

Acknowledgments

This work was supported by Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine (No. ZPKLPVM2017KFKT003), Key Laboratory of Prevention and Control Agents for Animal Bacteriosis (Ministry of Agriculture, No. KLPCAAB-2018-05) and National Natural Science Foundation of China (No. 31802208) to Chun Fang. We are grateful for the helpful comments of the reviewers.

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. Rabbits used for antibodies preparation were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

REFERENCES

1. Freitag NE, Port GC, Miner MD: Listeria monocytogenes - from saprophyte to intracellular pathogen. *Nat Rev Microbiol,* 7 (9): 623-628, 2009. DOI: 10.1038/nrmicro2171

2. Chen M, Cheng J, Zhang J, Chen Y, Zeng H, Xue L, Lei T, Pang R, Wu S, Wu H, Zhang S, Wei X, Zhang Y, Ding Y, Wu Q: Isolation, potential virulence, and population diversity of *Listeria monocytogenes* from meat and meat products in China. *Front Microbiol,* 10:946, 2019. DOI: 10.3389/fmicb.2019.00946

3. Roberts AJ, Wiedmann M: Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. *Cell Mol Life Sci,* 60 (5): 904-918, 2003. DOI: 10.1007/s00018-003-2225-6

4. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM: Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis,* 17 (1): 7-15, 2011. DOI: 10.3201/eid1701.P11101

5. Johansson J, Freitag NE: Regulation of Listeria monocytogenes virulence. *Microbiol Spectr,* 7 (4), 2019. DOI: 10.1128/microbiolspec.GPP3-0064-2019

6. Nguyen BN, Peterson BN, Portnoy DA: Listeriolysin O: A phagosome-specific cytolysin revisited. *Cell Microbiol,* 21 (3): e12988, 2019. DOI: 10.1111/cmi.12988

7. Ireton K, Rigano LA, Polle L, Schubert WD: Molecular mechanism of protrusion formation during cell-to-cell spread of Listeria. *Front Cell Infect Microbiol,* 4:21, 2014. DOI: 10.3389/fcimb.2014.00021

8. Smith JL, Liu Y, Paoli GC: How does *Listeria monocytogenes* combat acid conditions? *Can J Microbiol,* 59 (3): 141-152, 2013. DOI: 10.1139/cjm-2012-0392

9. Ryan S, Hill C, Gahan CG: Acid stress responses in *Listeria monocytogenes*. *Adv Appl Microbiol,* 65, 67-91, 2008. DOI: 10.1016/S0065-2164(08)00603-5

10. Ryan S, Begley M, Gahan CGM, Hill C: Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. *Environ Microbiol,* 11 (2): 432-445, 2009. DOI: 10.1111/j.1462-2920.2008.01782.x

11. Cheng C, Chen J, Fang C, Xia Y, Shan Y, Liu Y, Wen G, Song H, Fang W: *Listeria monocytogenes aguA1*, but Not aguA2, Encodes a Functional Agmatine Deiminase: Biochemical Characterization of its catalytic properties and roles in acid tolerance. *J Biol Chem,* 288 (37): 26606-26615, 2013. DOI: 10.1074/jbc.M113.477380

12. Cotter PD, Gahan CG, Hill C: A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol Microbiol,* 40 (2): 465-475, 2001.

13. Abram F, Starr E, Karatzas KA, Matlawska-Wasowska K, Boyd A, Wiedmann M, Boor KJ, Connally D, O'Byrne CP: Identification of components of the sigma B regulon in Listeria monocytogenes that contribute to acid and salt tolerance. *Appl Environ Microbiol,* 74 (22): 6848-6858, 2008. DOI: 10.1128/AEM.00442-08

14. Chen J, Fang C, Zheng T, Zhu N, Bei Y, Fang W: Genomic presence of gadD1 glutamate decarboxylase correlates with the organization of ascB-dapE internalin cluster in *Listeria monocytogenes*. *Foodborne Pathog Dis,* 9 (2): 175-178, 2012. DOI: 10.1089/fpd.2011.1022

15. Cheng C, Chen J, Shan Y, Fang C, Liu Y, Xia Y, Song H, Fang W: *Listeria monocytogenes* ArcA contributes to acid tolerance. *J Med Microbiol,* 62 (6): 813-821, 2013. DOI: 10.1099/jmm.0.055145-0

16. Fang C, Cao T, Shan Y, Xia Y, Xin Y, Cheng C, Song H, Bowman J, Li X, Zhou X, Fang W: Comparative genomic analysis reveals that the 20K and 38K prophages in *Listeria monocytogenes* serovar 4a strains Lm850658 and M7 contribute to genetic diversity but not to virulence. *J Microbiol Biotechnol,* 26 (1): 197-206, 2016. DOI: 10.4014/jmb.1504.04075

17. Fang C, Chen X, Liang X, Fang X, Gao K, Chen J, Gu Y, Yang Y: The effect of single amino acid substitution in *SecA2* on protein translocation and pathogenicity of *Listeria monocytogenes*. *Kafkas Univ Vet Fak Derg,* 25 (5): 665-672, 2019. DOI: 10.9775/kvfd.2018.21558

18. Cheng C, Jiang L, Ma T, Wang H, Han X, Sun J, Yang Y, Chen Z, Yu H, Hang Y, Liu F, Wang B, Fang W, Huang H, Fang C, Cai C, Freitag N, Song H: Carboxyl-terminal residues N478 and V479 required for the cytolytic activity of listeriolysin o play a critical role in *Listeria monocytogenes* pathogenicity. *Front Immunol,* 8:1439, 2017. DOI: 10.3389/fimmu.2017.01439

19. Cheng C, Dong Z, Han X, Wang H, Jiang L, Sun J, Yang Y, Ma T, Shao C, Wang X, Chen Z, Fang W, Freitag NE, Huang H, Song H: Thioredoxin a is essential for motility and contributes to host infection of *Listeria monocytogenes* via redox interactions. *Front Cell Infect Microbiol,* 7:287, 2017. DOI: 10.3389/fcimb.2017.00287

20. Radoshevich L, Cossart P: *Listeria monocytogenes*: Towards a complete picture of its physiology and pathogenesis. *Nat Rev Microbiol,* 16 (1): 32-46, 2018. DOI: 10.1038/nrmicro.2017.126

21. Gahan CGM, Hill C: *Listeria monocytogenes*: Survival and adaptation in the gastrointestinal tract. *Front Cell Infect Microbiol,* 4:9, 2014. DOI: 10.3389/fcimb.2014.00009

22. Feehily C, Finnerty A, Casey PG, Hill C, Gahan CG, O'Byrne CP, Karatzas KAG: Divergent evolution of the activity and regulation of the glutamate decarboxylase systems in *Listeria monocytogenes* EGD-e and 10403S: Roles in virulence and acid tolerance. *PLoS One,* 9 (11): e112649, 2014. DOI: 10.1371/journal.pone.0112649

23. Karatzas KAG, Brennan O, Heavin S, Morrissey J, O'Byrne CP: Intracellular accumulation of high levels of gamma-aminobutyrate by *Listeria monocytogenes* 10403S in response to low pH: uncoupling of gamma-aminobutyrate synthesis from efflux in a chemically defined medium. *Appl Environ Microbiol,* 76 (11): 3529-3537, 2010. DOI: 10.1128/AEM.03063-09

24. Cheng C, Dong Z, Han X, Sun J, Wang H, Jiang L, Yang Y, Ma T, Chen Z, Yu J, Fang W, Song H: *Listeria monocytogenes* 10403S arginine repressor ArgR finely tunes arginine metabolism regulation under acidic conditions. *Front Microbiol,* 8:145, 2017. DOI: 10.3389/fmicb.2017.00145

25. Cotter PD, Gahan CGM, Hill C: Analysis of the role of the *Listeria monocytogenes* F0F1 -AtPase operon in the acid tolerance response. *Int J Food Microbiol,* 60 (2-3): 137-146, 2000. DOI: 10.1016/S0168-1605(00)00305-6

26. Datta AR, Benjamin MM: Factors controlling acid tolerance of *Listeria monocytogenes*: Effects of nisin and other ionophores. *Appl Environ Microbiol,* 63 (10): 4123-4126, 1997.

27. Chen J, Chen F, Cheng C, Fang W: Prevalence of the *lmo0036-0043* gene cluster encoding arginine deiminase and agmatine deiminase systems in *Listeria monocytogenes*. *New Microbiol,* 36 (2): 187-192, 2013.

28. Karatzas KA, Suur L, O'Byrne CP: Characterisation of the intracellular-glutamate decarboxylase system: Analysis of its function, transcription and role in the acid resistance of various strains of *Listeria monocytogenes*. *Appl Environ Microbiol*, 78 (10): 3571-3579, 2012. DOI: 10.1128/AEM.00227-12

29. NicAogain K, O'Byrne CP: The Role of stress and stress adaptations in determining the fate of the bacterial pathogen *Listeria monocytogenes* in the food chain. *Front Microbiol,* 7:1865, 2016. DOI: 10.3389/fmicb.2016.01865

30. Pizarro-Cerda J, Cossart P: *Listeria monocytogenes*: Cell biology of invasion and intracellular growth. *Microbiol Spectr,* 6 (6), 2018. DOI: 10.1128/microbiolspec.GPP3-0013-2018

31. Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M: *Listeria monocytogenes* σ^B regulates stress response and virulence functions. *J Bacteriol,* 185 (19): 5722-5734, 2003.

32. Cotter PD, Ryan S, Gahan CG, Hill C: Presence of GadD1 glutamate decarboxylase in selected *Listeria monocytogenes* strains is associated with an ability to grow at low pH. *Appl Environ Microbiol,* 71 (6): 2832-2839, 2005. DOI: 10.1128/AEM.71.6.2832-2839.2005

33. Bowman JP, Hages E, Nilsson RE, Kocharunchitt C, Ross T: Investigation of the *Listeria monocytogenes* scott A acid tolerance response and associated physiological and phenotypic features via whole proteome analysis. *J Proteome Res,* 11 (4): 2409-2426, 2012. DOI: 10.1021/pr201137c

Kafkas Univ Vet Fak Derg
26 (2): 239-246, 2020
DOI: 10.9775/kvfd.2019.22854

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Comparison of Tenderness and Calpains Activity of Yak Meat in Different Ages During Postmortem Aging

Pengcheng WEN [1,a] Qiaoneng YANG [2,b] Weibing ZHANG [1,c] Min YANG [1,d]
Qi LIANG [1,e] Yaoyao JIAO [1,f] Yanli ZHU [1,g]

[1] College of Food Science and Engineering, Gansu Agricultural University, Lanzhou 730070, CHINA
[2] Information Center of Gansu Food and Drug Administration, Lanzhou 730070, CHINA
[a] ORCID: 0000-0002-5555-6482; [b] ORCID: 0000-0003-2034-2342; [c] ORCID: 0000-0001-5739-6702; [d] ORCID: 0000-0002-5157-4800
[e] ORCID: 0000-0001-8717-3892; [f] ORCID: 0000-0003-1741-1175; [g] ORCID: 0000-0001-8547-2610

Article ID: KVFD-2019-22854 Received: 20.06.2019 Accepted: 23.10.2019 Published Online: 28.10.2019

How to Cite This Article

Wen P, Yang Q, Zhang W, Yang M, Liang Q, Jiao Y, Zhu Y: Comparison of tenderness and calpains activity of Yak meat in different ages during postmortem aging. *Kafkas Univ Vet Fak Derg,* 26 (2): 239-246, 2020. DOI: 10.9775/kvfd.2019.22854

Abstract

Yak is a rare breed of resources in Tibetan Plateau, with high protein and low-fat content, and has other important application value. Yaks are managed by herders and slaughtered in various ages, changing from three years to twelve years of age, and there has a significant difference on meat tenderness in different ages. This study was conducted to investigate the effects of age at harvest, ageing time and the correlation analysis to meat tenderness calpains activity. The work may be used to establish a classification system and grading standards for yak meat. Four groups were established as fewer than 3 years-old, 3-5 years-old, 5-7 years-old and more than 7-years old. The results demonstrated that the content of tenderness has significantly different ($P<0.05$) in various ages and it increased and then decreased during postmortem aging. The muscle fiber diameter has decreased with the increase of slaughter age, while the MFI showed the contrary. The MFI increased with aging. The myofibrils' ultrastructure was completely disrupted. Calpains activity was significantly decreased ($P<0.05$) in the first 3 d and then decreased with aging. The calpains activity of yak meat in different ages has increased and then decreased with the different ages. The nitration of μ-calpain promoted its ability in degrading a part of myofibrillar proteins and the degrading of myofibrillar proteins related to the tenderness of meat.

Keywords: *Yak meat, Ages, Postmortem aging, Meat tenderness, Calpains*

Farklı Yaşlardaki Yaklara Ait Etlerin Postmortem Olgunlaştırma Sürecinde Yumuşaklık ve Kalpain Aktivitelerinin Karşılaştırılması

Öz

Yak, Tibet Platosu'nda yaşayan, yüksek protein ve düşük yağ içerikli etinin yanı sıra uygulamada önemli değere sahip diğer nitelikleri ile nadir bir cinstir. Çobanlar tarafından idare edilen Yakların, 3-20 arasında değişen yaşlarda kesimleri gerçekleştirilir ve bu yaş farklılıkları etin yumuşaklığı üzerinde belirgin bir fark oluşturur. Bu çalışma, kesim yaşı, olgunlaştırma zamanı ve korelasyon analizinin etin yumuşaklık kalpain aktivitesine etkisini araştırmak üzere yürütülmüştür. Çalışma sonuçları, Yak etinin sınıflandırma sistemi ve derecelendirme standardının oluşturulmasında yararlı olabilir. Araştırmada 3 yaşından küçük, 3-5 yaş arası, 5-7 yaş arası ve 7 yaşından büyük olmak üzere 4 grup oluşturulmuştur. Bulgular, yumuşaklık göstergesi olan parametrelerin yaş gruplarına göre belirgin bir şekilde değiştiğini ($P<0.05$), postmortem olgunlaştırma sürecinde önce artıp sonra azaldığını göstermiştir. Kesim yaşı arttıkça kas lifi çapının azaldığı, ancak MFI için tam tersi bir durumun sözkonusu olduğu belirlenmiştir. MFI, olgunlaştırma ile birlikte artış göstermiştir. Kas liflerinin ultrayapısı tamamıyle bozulmuştur. Kalpainlerin aktivitesi ilk 3 günde belirgin biçimde azalmış ($P<0.05$) ve ardından olgunlaştırma süresince azalmıştır. Farklı yaşlardaki Yaklara ait etlerdeki kalpainlerin aktiviteleri artmış ve sonrasında yaş farklılıklarına bağlı olarak azalmıştır. μ-calpain nitrasyonu miyofibriler proteinlerin parçalanmasını hızlandırmış, böylece et yumuşaklığı üzerine etki göstermiştir.

Anahtar sözcükler: *Yak eti, Yaş, Postmortem olgunlaştırma, Et yumuşaklığı, Kalpainler*

INTRODUCTION

The yak meat is famous for its low fat, high protein, fine texture and rich amino acids compared to beef [1]. Consequently, more and more people choose the green and non-pollution yak meat [2]. Nowadays, on the source of Yak meat, ongoing research is underway on the yak meat composition [3,4]. Male Yak meat is rich in phosphorus and

İletişim (Correspondence)
+86 13639315437
wenpch_gau@163.com

calcium, compared to female meat as well as cattle meat [5]. Wild Yak meat is rich in iron and various essential minerals [5]. Ageing is one effective way to enhance tenderness and meat tenderness is one of the most important factors in terms of consumer purchase [6,7]. Many researchers study Yak meat quality, and they are focused on the effect of the breed of beef, feeding time, gender, trophic levels, and feed additives on tenderness [8,9]. Postmortem tender process, color stability and water holding capacity of yak meat were also studied [9,10]. Meat processing and meat quality of yak in different ages during postmortem aging were also investigated [9,10]. However reports about the difference of yak meat tenderness and the mechanism are very few. Therefore, the aims of this research were to demonstrate the difference of meat tenderness and calpains activity of yak in different ages during postmortem aging.

MATERIAL and METHODS

Materials and Reagents

Sixty healthy yaks, selected after examination by a veterinarian, were used from Maqu country in Gannan. The fasting 24 h, 12 h water deprivation was compiled before yaks slaughtered. *Longissimus dorsi* muscle was removed from the carcasses immediately after slaughter and cut into chops with an average weight of 200 g, vacuum packed into pouches, taken to the laboratory under refrigerated condition. The samples were refrigerated at 4°C for 0, 1, 2, 3, 4, 5, 6 and 7 days, respectively. The shear force analysis was made in fresh meat samples. The samples tested for muscle fiber diameter are fixed in 10% formaldehyde solution, and those tested for myofibrils ultrastructure are fixed in 3% glutaraldehyde solution. Other samples were stored at -80°C until tested for muscle fiber diameter, myofibrillar fragmentation index, myofibrils ultrastructure and calpain activity. There were four experimental groups: ≤3 years, 3-5 years, 5-7 years, >7 years 4 groups.

Tenderness Measurement

Shear Force: Samples were packed in plastic bags and submitted for cooking in a water bath until the internal temperature reached the value of 75°C, maintain 15 min and then cool down to room temperature. Samples were cut into 1 cm^3, and analyzed on a texturometer C-LM4 [11].

Muscle Fiber Diameter: Formaldehyde fixed samples were taken out, stripped of muscle fiber, and then measured by micrometer at 400 times magnification. In each sample 100 muscle fibers were measured, and the averages were calculated.

Myofibrillar Fragmentation Index (MFI): MFI was determined according to the procedures described by Kriese et al.[12]. One gram muscle sample, free of external fat and visible connective tissue, were homogenized for 30 s in 20 mL of MFI buffer (100 mM KCl, 20 mM potassium phosphate, 1 mM EDTA, 1 mM $MgCl_2$ and 1 mM NaN_3 at pH 7.0). 1 mM EDTA, 1 mM $MgCl_2$ and 1 mM NaN_3 at pH 7.0). The homogenate was centrifuged at 10×10^3 rpm for 15 min at 4°C, the supernatant discarded and pellet resuspended in 20 mL of the MFI buffer and centrifuged at 10×10^3 rpm for 15 min at 4°C. The supernatant was discarded and the pellet suspended in 10 mL of the same MFI buffer. The myofibril suspension was poured through a strainer to remove connective tissue, and then assayed for protein concentration using the biuret method. Aliquots of the suspensions were diluted in the MFI buffer to a final protein concentration of 0.5 mg/mL. The diluted protein suspension was poured into a cuvette and the absorbance at 540 nm was immediately measured with a spectrophotometer. The MFI was expressed as A540 nm×200.

Myofibrils Ultrastructure: Myofibrils ultrastructure was determined according to the procedures of Xu et al.[13]. The samples were cut into 0.5×0.5×0.5 cm small squares, and put in 3% glutaraldehyde solution (0.1 mol/L, pH 7.3), fixed and stored 3 days or longer at 0~4°C. Then, the samples were rinsed with 0.1 mol/L phosphate buffer solution 10 min and then fixed 1 h in 1% osmium tetroxide (0~4°C). Samples were rinsed with 0.1 mol/L phosphate buffer 10 min. With a concentration of respectively 30%, 50%, 70%, 90% and 100% ethanol gradually elute and with 100% ethanol dehydration once again. Samples were processed with Epon 812 epoxy resin and cut into 50 mm flake and then dyed with uranyl acetate and lead citrate. the samples were observed under a transmission electron microscope, and photographed.

Calpain Activity

• *Crude Calpain Activity Determination*

Crude Enzyme Extraction: Extraction, separation and assay of calpains were done according to the method described by Delgado et al.[14]. Briefly, the samples from each of the yaks were removed after death or after 1, 2, 3, 4, 5, 6 and 7 days of postmortem storage. The samples were trimmed free from visible fat and connective tissue and were homogenized at 4°C and 1 L extraction liquid was added (pH 8.3, 100 mmol/L Tris-HCl, 10 mmol/L EDTA, 0.05% MCE, 100 mg/L, Ovomucoid inhibitor, 2 mmol/L PMSF, 6 mg/L Leupeptin) then centrifuged (4°C, 10000 r/min 1.5 h), the precipitate was discarded, and the volume of the supernatant was recorded and the supernatant was salted out between 0 and 45% ammonium sulfate saturation. Then the sediment protein was dialyzed (pH 7.5, 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.1% MCE). After dialysis, the dialyzed extract was clarified by centrifugation at 4°C, 10.000 r/min for 1.5 h and filtered with 0.45 μm needle filter.

Crude Activity Measurement: The procedure to determin crude activity was a modification of the method described by Koohmarie [15] and Huang Ming [16]. The samples were taken 1 mL and the reaction mixture (100 mmol/L Tris,

10 mmol/L MCE, 5 mg/mL Casein-Hammerstein, the pH 7.5 with acetic acid) was added. The reaction was started adding 100 µL 0.1 mol/L $CaCl_2$, and it was stopped after 30 min with 2 mL of 5% TCA and the mixture was centrifuged at 6.000 g for 30 min. The absorbance of the supernatant was read at 278 nm.

• µ-Calpain in vitro Nitration of Myofibrillar Protein Degradation

The Separation and Purification of µ-Calpain: µ-Calpain was purified using DEAE-Sepharose-FF, selection the highest activity of crude enzyme calpain, and use of equilibrated solution TEMA (4ºC, pH7.5, 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.1% MCE, 1 mmol/L NaN_3), then TEMA solution was used to elute with a concentration of 0~500 mmol/L NaCl, until the absorbance value of sample liquid less than 0.1 at A280 nm.

Myofibrillar Protein Extraction: The procedure to extract myofibrillar rotein was a modification of the method described by Liu [17]. The longissimus sample trimmed, cut into pieces, 10 volumes salt solution was added (20 mmol/L potassium phosphate buffer, 0.1 mol/L KCl, 2 mmol/L $MgCl_2$, 2 mmol/L EGTA, pH 6.8), and homogenated 10 s (13.000 r/min) then centrifuged 10 min (1000 r/min) at 4ºC, and the supernatant was discarded. Using 8 volumes salt solution rinse the precipitation, and using the 8 volumes salt solution (20 mmol/L potassium phosphate buffer, 0.1 mol/L KCl, 2 mmol/L $MgCl_2$, 2 mmol/LEGTA, 1% Triton-100, PH 6.8) what contain 1% Triton-100, then rinsing with 8 volumes 100 mmol/L KCl. After rinsing, the precipitate was dissolved in the incubation buffer without DTT (5 mmol/L Hepes, 100 mmol/L NaCl, 0.1% Chaps, 5 mmol/L NaN_3, pH 6.5) in.

Protein Concentrations Determination: Coomassie Brilliant Blue

ONOO- Preparation: The procedure to prepare ONOO- was a modification of the method described by Beckman et al.[18]. Mixe the dilution 100 mL containing 12.5 mL 0.6 mol/L HCl and 15 mL 0.7 mol/L H_2O_2, and prepared 100 mL 0.6 mol/L $NaNO_2$, then filtered to brown reagent bottle at -20ºC cryo- preservation. Before each test, with 1 mol/L NaOH diluted ONOO-, according to 302 nm absorbance values to determine the concentration of ONOO- (ε302 nm = 1670 L/mol • cm).

µ- Calpain In Vitro Nitration: Active enzyme was randomly divided into seven groups, with six different concentrations of peroxynitrite solution, to produce different levels of nitration of ONOO-, every groups were processed 30 min at 37ºC.

Nitration µ-Calpain Incubated Myofibrils: After the nitration µ-calpain oxide ended in different conditions, purified myofibrils and nitration µ-calpain were mixed immediately.

Incubation Conditions: 600 µL of myofibrillar protein (10 mg/mL), 15 µL µ-calpain, 37ºC water bath for 30 min. After the incubation, the samples were placed in ice immediately for stopping the reaction and an appropriate amount of sample reagent solution (0.6 mL 1 mol/LTris-HCl pH6.8, 5 mL 50% glycerin, 2 mL 10% SDS, 0.5 mL MCE, 1 mL BPB, 0.9 mL distilled water)was added in water bath at 100ºC. Then the samples were stored at -80ºC.

SDS-PAGE Electrophoresis Analysis: Electrophoresis conditions: separation gel concentrations of 12%, spacer gel concentrations of 5%, voltage 80v. With Coomassie Brilliant Blue R-250 staining 1 h after electrophoresis, and decolorization processing placed a night.

Data Analysis

Processing data using Microsoft Excel 2007; Using SPSS 19.0 for data analysis and significant pearson correlation analysis.

RESULTS

Shear Force

Shear force is the most important quality index of meat, which reflect the tenderness of meat. Lower the value of the meat is, tenderer it is. The results of the shear force in the mature process of yak in different ages during postmortem aging are shown in *Table 1*.

The changes of shear force for yak meat during postmortem aging are shown in *Table 1*. Significant increase and than decrease in shear force was found in the first 3 day and 3-7 days after aging, respectively. Among the different ages, yak meat tenderness significantly differed ($P<0.05$). The initial gradual increase in meat shear force is a typical of muscle going into rigor mortis, the decrease there after is what could be expected as meat is aged.

Table 1. *Difference in Share force values (kgf) during postmortem aging of yak meat in different ages*

Index	Mature Time (d)					
	0	1	2	3	5	7
≤3 years	$3.45\pm0.29^{a,B}$	$4.60\pm0.28^{a,C}$	$5.22\pm0.13^{a,D}$	$6.92\pm0.46^{a,DE}$	$5.31\pm0.35^{a,D}$	$2.87\pm0.23^{a,A}$
3~5 years	$4.11\pm0.66^{b,A}$	$5.02\pm0.65^{a,B}$	$7.21\pm0.18^{b,C}$	$9.38\pm0.03^{b,D}$	$6.60\pm0.35^{b,E}$	$3.82\pm0.27^{b,A}$
5~7 years	$5.31\pm0.65^{c,B}$	$7.60\pm0.73^{b,C}$	$9.40\pm0.54^{c,D}$	$10.40\pm0.70^{c,E}$	$7.98\pm0.02^{c,C}$	$4.04\pm0.26^{b,A}$
>7 years	$6.49\pm0.33^{d,B}$	$7.55\pm0.74^{b,C}$	$9.19\pm0.49^{c,D}$	$11.30\pm1.25^{d,E}$	$8.32\pm0.66^{c,C}$	$5.26\pm0.98^{c,A}$

Table 2. *Difference in muscle fiber diameter(μm) during postmortem aging of yak meat in different ages*

Index	Mature Time (d)					
	0	1	2	3	5	7
≤3 years	49.71±0.63^{a,A}	48.45±0.67^{a,B}	47.87±0.52^{a,C}	45.39±0.50^{a,D}	41.97±0.49^{a,E}	40.62±0.68^{a,F}
3~5 years	55.38±0.46^{b,A}	54.98±0.55^{b,A}	54.03±0.82^{b,B}	51.88±0.48^{b,C}	48.38±0.52^{b,D}	46.78±0.43^{b,E}
5~7 years	59.88±0.49^{c,A}	58.54±0.14^{c,B}	57.43±0.80^{c,C}	53.78±0.45^{c,D}	50.01±0.90^{c,E}	48.26±0.98^{c,F}
>7 years	63.33±0.98^{d,A}	61.32±0.66^{d,B}	60.65±0.53^{d,C}	56.89±0.57^{d,D}	53.21±0.44^{d,E}	51.08±0.43^{d,F}

Table 3. *Difference in MFI during postmortem aging of yak meat in different ages*

Index	Mature Time (d)					
	0	1	2	3	5	7
≤3 years	35.03±0.64^{a,A}	61.75±0.76^{a,B}	100.02±0.66^{a,C}	122.47±0.42^{a,D}	132.78±0.02^{a,D}	137.25±0.41^{a,D}
3~5 years	29.54±0.31^{b,A}	42.95±0.31^{b,B}	79.13±0.23^{a,C}	108.68±0.42^{b,D}	111.21±0.64^{b,DE}	115.07±0.62^{b,E}
5~7 years	21.03±0.21^{c,A}	30.11±0.32^{c,B}	64.24±0.07^{b,C}	97.32±0.03^{c,D}	102.45±0.42^{c,DE}	105.24±0.57^{c,E}
>7 years	13.54±0.41^{d,A}	22.28±0.42^{d,B}	56.44±0.25^{c,C}	77.24±0.67^{d,D}	83.38±0.75^{d,DE}	86.34±0.24^{d,E}

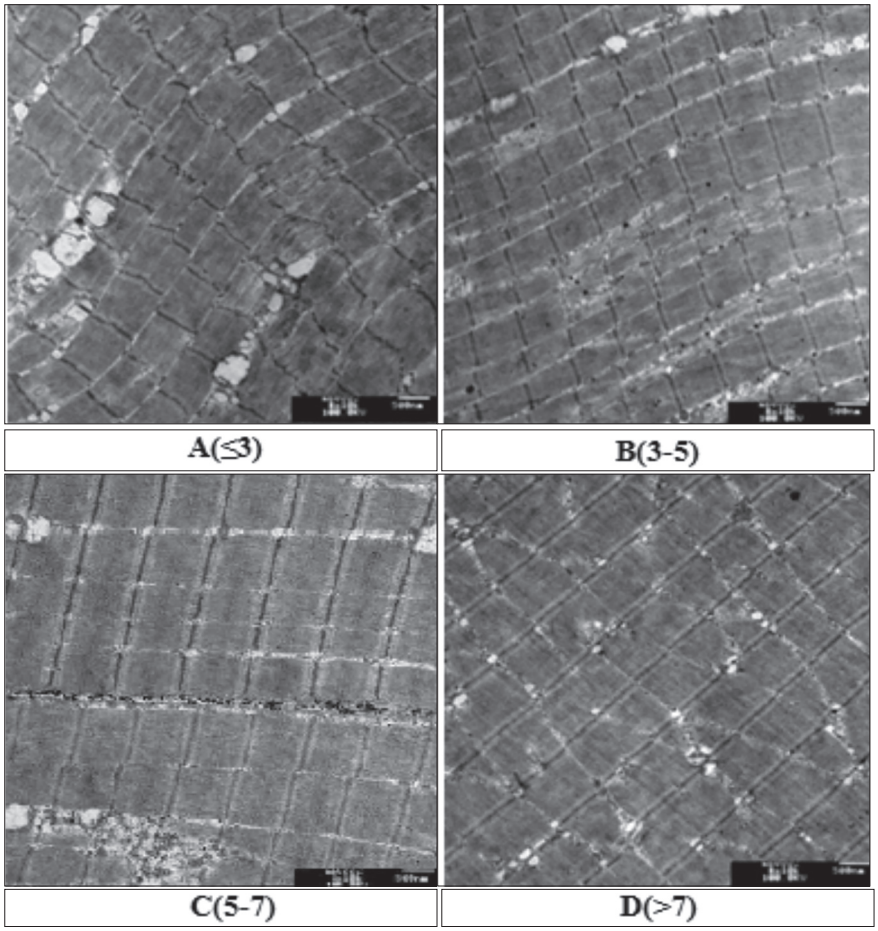


Fig 1. Myofibrils ultrastructure of yak meat in different ages (×10000)

Muscle Fiber Diameter

Muscle fiber diameter as an important index for muscle tenderness, and have a great impact on muscle tenderness. The measurement results of different ages during postmortem aging of muscle fiber diameters as shown in *Table 2*.

The changes of shear force for yak meat during postmortem aging are shown in *Table 2*. Significant decrease in muscle fiber diameter was found 0-7 days after ageing ($P<0.05$), and also significant differences between different ages were seen ($P<0.05$).

Muscle Fiber Fragmentation Index

The changes of MFI for yak meat during postmortem aging are shown in *Table 3*. It shows a significant increase during 0-7 days and then remains constant, and there iare also significant differences between the different ages ($P<0.01$). The higher the age, the lower the MFI of the samples.

Myofibrils Ultrastructure

Fig. 1 shows myofibrils ultrastructure of samples at different ages by transmission electron microscopy. Myofibrils ultrastructure are consistent on the form in different ages, and Z- line clearly. Between two Z lines of a sarcomere, the longer the sarcomere, the better the tenderness. As we can see from the *Fig. 1*, 3 years old yak meat myofibrillar ultrastructure sarcomere are relatively long, and 3 to 5 years old we can see the sarcomere are clearly shorter; 5 to 7 years and beyond 7 years old the sarcomere length compare to the 3 to 5 years old is even shorter.

Table 4. *Correlations analysis between age and myofibrils ultrastructure indicators*

Index	Age	Muscle Fiber Diameter	MFI
Age	1	0.737**	-0.387
Muscle fiber diameter		1	0.714*
MFI			1

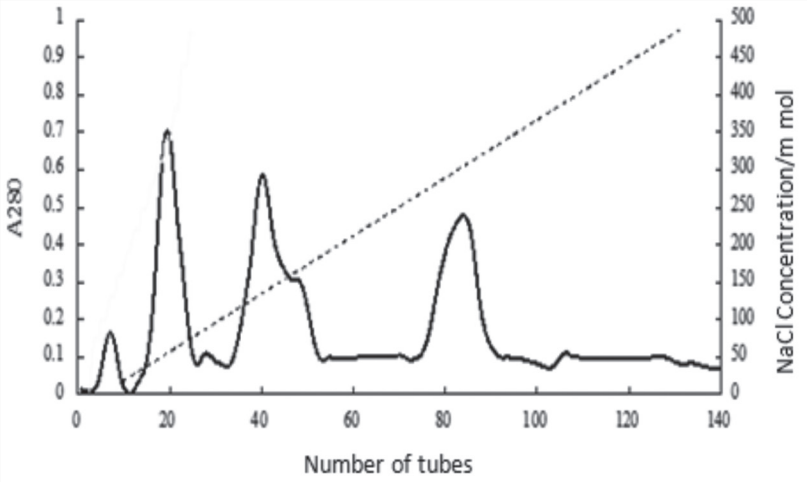


Fig 2. Elution profile of calpain system loaded onto DEAE-Sepharose-FF column

in calpain activity was found 0-2 days after ageing (P<0.05), and the rate of decline slowed down after 3 days. The crude calpain activity was initially increased, and it subsequently decreased in 0 day after aging, it is related to the body metabolism of different ages. Calpains activity of yak meat in different age decreased in all the tested postmortem aging groups. On one hand, it is due to lack of the calcium activated enzyme (consumed), chaplains can degrades the other proteins, and also can degrade itself.

Correlation Analysis Between Calpain Activity and Tenderness

As it can be seen from *Table 6*, shear force and muscle fiber diameter had a significant positive correlation (P<0.05) during postmortem aging. Shear, MFI and muscle fiber diameter and calpains activity correlation significantly (P<0.01). The shear force can be responsiable from the tenderness of the yak meat directly, and

Table 5. *Difference in calpains during postmortem aging of yak meat in different ages*

Index	Mature Time (d)					
	0	1	2	3	5	7
≤3years	0.738±0.019^{a,A}	0.549±0.023^{a,B}	0.249±0.064^{a,C}	0.191±0.045^{a,CD}	0.161±0.063^{a,D}	0.145±0.029^{a,D}
3-5years	0.830±0.034^{b,A}	0.628±0.055^{b,B}	0.380±0.053^{a,C}	0.273±0.045^{a,D}	0.247±0.074^{a,D}	0.231±0.051^{a,D}
5-7years	0.823±0.045^{c,A}	0.615±0.021^{c,B}	0.312±0.060^{a,Cb}	0.261±0.037^{b,C}	0.238±0.092^{b,C}	0.224±0.064^{b,C}
>7years	0.658±0.066^{c,A}	0.506±0.014^{c,B}	0.256±0.034^{b,C}	0.213±0.036^{b,C}	0.196±0.023^{b,C}	0.179±0.097^{b,C}

Table 6. *Correlation analysis between tenderness indexes of yak meat*

Index	Shear Force	MFI	Muscle Fiber Diameter	The Crude Enzyme Activity
Shear force	1	-0.012	0.489*	-0.291
MFI		1	-0.822**	-0.881**
Muscle fiber diameter			1	0.533**
The crude enzyme activity				1

Correlation Between Age, Muscle Fiber Diameter and the MFI

The correlations between the age, and the muscle fiber diameter and the MFI are shown in *Table 4*. Age and muscle fiber diameter were significantly positivly correlated (P<0.01), and MFI was negatively correlated. This shows that age has a greater impact on muscle tenderness. The bigger the yak's age, the smaller fiber diameter of the muscle, the smaller the MFI value, and the less tenderness of samples.

Changes in calpain activity for yak meat during post-mortem aging are shown in *Table 5*. A significant decrease

four indicators (shear force, MFI muscle fiber diameter, and the crude enzyme activity) can be used as indicators of the evaluation of yak meat tenderness.

In vitro Nitro μ-Calpain of Yak Meat Myofibrillar Protein Degradation

DEAE-Sepharose-FF ion exchange column was used to separate and purify the calpains, and Calpastatin, μ-calpain, m-calpain are separated completely *(Fig. 2)*. Calpastatin in the range of 30~110 mmol/L NaCl concentration was eluted, μ-calpain in the range of 130~180 mmol/L NaCl concentration was eluted and m-calpain in the range of

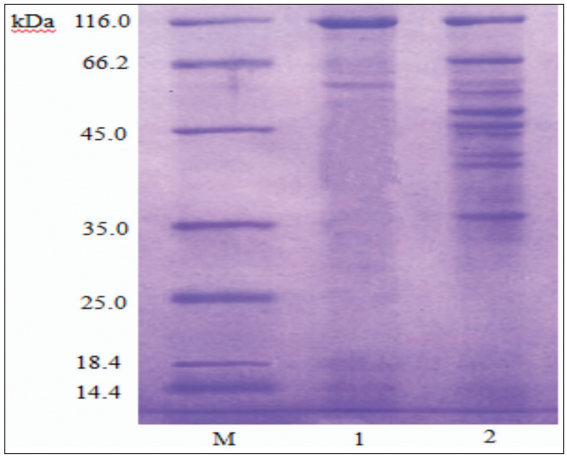


Fig 3. SDS-PAGE analysis of degradation of myofibrillar proteins incubated with μ-calpain nitrationed at different levels. M: Marker; 1: Control; 2: 0.2 mmol/LONOO-; 3: 0.4 mmol/LONOO-; 4: 0.6 mmol/LONOO-; 5: 0.8 mmol/L ONOO-; 6: 1.0 mmol/L ONOO-; 7: 1.5 mmol/L ONOO-

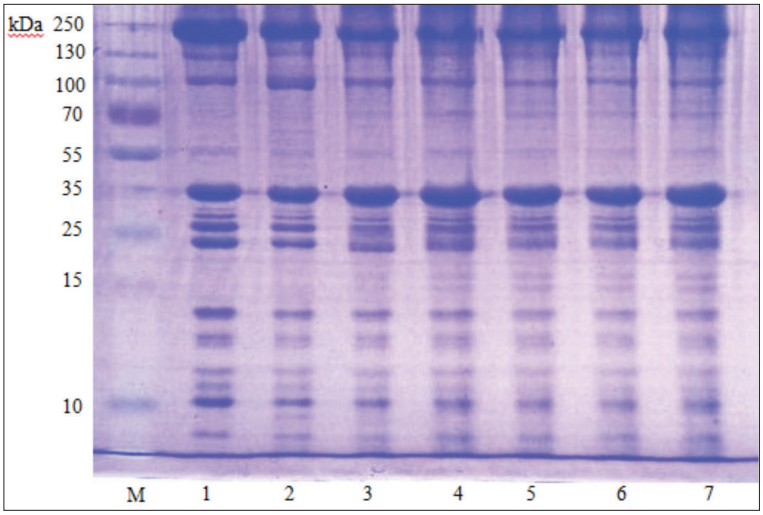


Fig 4. SDS-PAGE of purified μ-calpain. M: Marker; 1: 0~500 mmol/LNaCl; 2: 0~330 mmol/LnaCl

Table 7. *Changes in μ -calpain of different degradation*

Treatments	1	2	3	4	5	6	7
μ-calpains Activity	0.133±0.002^{a}	0.163±0.006^{b}	0.182±0.002^{c}	0.208±0.002^{d}	0.235±0.007^{e}	0.268±0.004^{f}	0.281±0.008^{g}

260~330 mmol/L NaCl concentration eluted,the collected part what has μ-calpains activity was concentrated.

As can be seen from *Fig. 3*, the degradation ability of μ-calpain to myofibrillar protein was enhanced by its nitration. Compared to the control group, after the incubation of 1.0 mmol/L $ONOO^-$ and 1.5 mmol/L ONOO- treated μ-calpain and myofibrillar protein, two protein bands appeared at relative molecular mass of 15 kDa and 17 kDa. After the concentration of the nitrating reagent is reduced, the above-mentioned two bands disappeared. The protein bands at relative molecular mass of 250 kDa, 100 kDa, and 10 kDa gradually became lighter in pace with increasing the concentration of the nitrating reagent *(Fig. 4)*, but the protein bands of 35 kDa gradually strengthened as the concentration of the nitrating reagent increased. These changes are identical to the changes in μ-calpain activity after nitration *(Table 7)*.

DISCUSSION

From this study, it can be seen that both age and slaughter handling of yak meat muscle fiber diameter have a greater impact. If we grade the meat according to their age which is postmortem aging, and the use of appropriate ripening conditions will improve muscle tenderness.

The results showed that the active ingredient collected on SDS-PAGE showing a single band of molecular weight of about 100kDa after DEAE-Sepharose-FF ion exchange column purification *(Fig. 4)*, it is the same to Hu Peng et al.[19] measured μ-calpain relative molecular weight of about 100kDa.

The value of shear force for yak meat during postmortem aging in the 0 day were 3.45 kgf, 4.11 kgf, 5.31 kgf, 6.49 kgf, these data showed that as the age increases the tenderness decreases. But after 7 days aging, the shear force value significantly decreased to 16.81%~22.01%. In different ages, the results were as similar to the findings of Hu Peng et al.[19]. Shear force was first increased and then decreased during postmortem aging, since sarcomere shortening leads to the rigor stage, and then muscle structure was destroyed under some endogenous enzymes, and the tenderness was increased [20].

Muscle fiber diameter as an important index for muscle tenderness, have a great impact on muscle tenderness. Light et al.[21] studied the different parts of the beef connective tissue content and features, and found that the more tender of the sample meat with a smaller diameter muscle fibers. Endomysium and muscle fibre are separated, and it lost with droplets during postmortem aging, this lead to reduces in fiber diameter value in yak meat in different age, after 7 days ($P>0.05$). Hence, postmortem aging can improve the tenderness of the yak meat.

Myofibrillar fragmentation index reflects the degree of myofibrillar protein degradation and muscle fiber structure is destroyed. The larger MFI, the better muscle tenderness, and myofibril structure damaged severely. In this study, MFI significantly increased in 0-3 day after slaughter ($P<0.05$), and its rise velocity and amplitude decreased and stabilized after 3 days, which was due to consumption of *in vivo* protease, and hence decreased activity, and the muscle fibrillin was no longer degraded [22].

The protein constituting the myofibrillar skeleton was destroyed by endogenous enzymes, myofibrillar was broken and degraded at Z-line and this change as an important indicator of muscle tenderness during post-mortem aging [23,24]. In this study, the myofibrillar ultra-structure was not significantly different in morphological point of view and Z-line clear. This test measured that with the age increases, the sarcomere length are shortened, which leads to aging of yak meat tenderness, and palatability greatly worsen. As it can be seen from the analysis of the correlation between the microstructure index, age and muscle fiber diameter, MFI values showed a highly significant correlation ($P<0.01$), it can be said that, when the age of yak is older, muscle fiber diameter is thicker and the MFI value is smaller, so the tenderness will decreased.

Tenderness is the most important indicator for meat quality evaluation, and the degradation of skeleton protein is a major factor to improved tenderness during postmortem aging. The present study has demonstrated that the calpain system plays a major role in postmortem muscle tenderness [25,26]. Calpain as a proteolytic enzyme, which is capable of hydrolyzing protein composition Z-line, it affects tropomyosin, M line protein, troponin T and so on [27]. In muscle tissue, calpains are divided into μ-calpain and m-Calpain, and μ-calpain is considered to be closely related to the meat tenderness during postmortem. The μ-Calpain activity decrease is a sign of hydrolysis [28]. During postmortem aging, muscle sarcoplasmic reticulum and mitochondria rupture, and Ca^{2+} is released, then calpains are activated and cause degradation of myofibrillar cytoskeletal proteins. Therefore, this process promotes increased tenderness [29].

The study found that the crude calpain activity increase and decrease in different age yak meat after aging. This may be because, the activity of calpains was lower before physiological maturity and when the aging begins, slow metabolic rate slowes, calpains activity is also reduced [30].

As it can be seen from the correlation analysis between the calpains activity and tenderness, shear forces, MFI and muscle fiber diameter and calpains activity correlated significantly ($P<0.01$). During postmortem aging, muscle sarcoplasmic reticulum and mitochondria rupture, and Ca^{2+} is released, then activation of calpains act on muscle fiber skeleton protein and make the muscle fibers weaken, thus it is improving the yak meat tenderness and playing a significant role in maturity and tenderness [15], Pringle [31] studied the different breeds of cattle, and found that the activity of calpains associated with tenderness. Cheng et al.[32] found that MFI and calpains activity can be used as important indicators on pork tenderness.

Oxidation phenomenon is widespread during postmortem aging and meat storage process. Some scholars think oxidative stress leads to protein oxidation [33] and oxidative stress, including reactive oxygen species; reactive oxygen species (ROS) and reactive nitrogen radicals (RNS). Some studies demonstrated that ROS on oxidative protein results in protein polymerization, crosslinking and forming groups and amino acid derivatives, these structural changes can increase susceptibility to proteolytic enzyme protein, thereby accelerating protein degradation, and reactive nitrogen radicals (RNS) allows protein tyrosine nitration reaction and produce 3-nitro-tyrosine (nitrotyrosine, NT) [33,34]. Studies have found that nitration of tyrosine residues can accelerate the proteolytic enzyme degradation of certain proteins, and the degradation rate associated with their degree of nitrification [34]. Thereby, nitration may changes the center of calpain active group and also change the degradation of myofibrillar proteins. Consequently, the study on nitration effects calpains is necessary.

This study demonstrated that nitration increases the activity of μ-calpain and promotes the degradation of myofibrillar proteins. There have been studies showning that μ-calpain activity in beef skeletal muscles increase with the degree of degradation [35]. Nitration is increasing the activity of μ-calpain, it probably because nitration μ-calpain inhibit their degradation. Myofibrillar protein degradation is believed to have a close relationship with the tenderness, it can be said that nitration to μ-calpain may improve muscle tenderness to some extent. However, this test mainly used isolated purified myofibrillar protein as a substrate, and is distinct with internal environment of meat. Therefore, the research of the mechanism about nitration of the postmortem meat tenderness are further needed.

Age and postmortem aging time had a significant effect on the tenderness of yak meat. After aging 7 days, shear force value is significantly decreased as compared to slaughter the first (0) day. The older of yak, the muscle fiber diameter thicker, and the sarcomere length shorter, the degree of fragmentation lower. After aging 7 days, muscle fiber diameter is thinner and the tenderness increases. Calpains activity was increased and then decreased during postmortem aging. Calpains activity was significantly decreased after postmortem before 2 days, and then decreased slowly. In vitro nitro μ-Calpain can improve this enzyme degradation of myofibrillar proteins, and μ-Calpain after nitration may improve muscle tenderness.

Acknowledgements

We gratefully acknowledged this work is supported by Discipline construction fund (GAU-XKJS-2019-xx), Fund for Fu Xi young talent training program (Lanzhou, China; Gaufx-02Y01), Young graduate student guidance fund support fund (Lanzhou, China; GAU- QNDS-201717).

REFERENCES

1. Mishra BP, Mishra J, Pati PK, Rath PK: Dehydrated meat products: A review. *Int J Livest Res,* 7, 10-22, 2017. DOI: 10.5455/ijlr.20170812035616

2. Lopa B, Herbert WO: Yak's Biochemistry contain the factors that make it possible for human habitation on the top of the world in central Asia. *Biomed J Sci Tech Res,* 1 (5): 1325-1327, 2017. DOI: 10.26717/BJSTR.2017.01.000430

3. Bai B, Hao L, Chai S, Niu J, Wang W, Liu S: Progress in understanding meat quality characteristics of yak. *Food Sci*, 35, 290-296, 2014.

4. Hao L, Yang X, Huang Y, Hocquette JF, Bryant RH, Xun W, Niu J, Sun L, Chai S, Ding L, Long R, Liu S: Using mineral elements to authenticate the geographical origin of Yak meat. *Kafkas Univ Vet Fak Derg*, 25 (1): 93-98, 2019. DOI: 10.9775/kvfd.2018.20366

5. Luo XL, Tong ZB, Wei YP, Zhao XQ: Meat characteristics of Qinghai yak and semi-wild yak. *Anim Sci J*, 77 (2): 230-234, 2006. DOI: 10.1111/j.1740-0929.2006.00342.x

6. Kim JH, Kim DH, Ji DS, Lee HJ, Yoon DK, Lee CH: Effect of aging process and time on physicochemical and sensory evaluation of raw beef top round and shank muscles using an electronic tongue. *Korean J Food Sci Anim Resour,* 37 (6): 823–832, 2017. DOI: 10.5851/kosfa.2017.37.6.823

7. Sosin-Bzducha E, Puchała M: Effect of breed and aging time on physicochemical and organoleptic quality of beef and its oxidative stability. *Arch Anim Breed,* 60, 191-198, 2017. DOI: 10.5194/aab-60-191-2017.

8. Cafferky J, Hamill RM, Allen P, O'Doherty JV, Cromie A, Sweeney T: Effect of breed and gender on meat quality of m. longissimus thoracis et lumborum muscle from crossbred beef bulls and steers. *Foods*, 8 (5): 173, 2019. DOI: 10.3390/foods8050173

9. Ramayo-Caldas Y, Renand G, Ballester M, Saintilan R, Rocha D: Multi-breed and multi-trait co-association analysis of meat tenderness and other meat quality traits in three French beef cattle breeds. *Genet Sel Evol*, 48:37, 2016. DOI: 10.1186/s12711-016-0216-y

10. Li S, Tang S, Yan L, Li R: Effects of microwave heating on physicochemical properties, microstructure and volatile profiles of yak meat. *J Appl Anim Res*, 47, 262-272, 2019. DOI: 10.1080/09712119.2019.1624553

11. Wiklund E, Kemp RM, LeRoux GJ, Li Y, Wu G: Spray chilling of deer carcasses - Effects on carcass weight, meat moisture content, purge and microbiological quality. *Meat Sci*, 86 (4): 926-930, 2010. DOI: 10.1016/j.meatsci.2010.07.018

12. Kriese PR, Soares AL, Guarnieri PD, Prudencio SH, Ida E, Iza I, Shimokomaki M: Biochemical and sensorial evaluation of intact and boned broiler breast meat tenderness during ageing. *Food Chem,* 104 (4): 1618-1621, 2007. DOI: 10.1016/j.foodchem.2007.03.003

13. Xu PT, Li Q, Sheng JJ, Chang H, Song Z, Yu ZB: Passive stretch reduces calpain activity through nitric oxide pathway in unloaded soleus muscles. *Mol Cell Biochem,* 367 (1-2): 113-124, 2012. DOI: 10.1007/s11010-012-1325-8

14. Delgado EF, Geesink GH, Marchello JA, Goll DE, Koohmaraie: The calpain system in three muscles of normal and callipyge sheep. *J Anim Sci*, 79 (2): 398-412, 2001. DOI: 10.2527/2001.792398x

15. Koohmaraie M, Geesink GH: Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpainsystem. *Meat Sci*, 74, 34-43, 2006. DOI: 10.1016/j.meatsci.2006.04.025

16. Huang J, Forsberg NE: Role of calpain in skeletal-muscle protein degradation. *Proc Natl Acad Sci USA*, 95, 12100-12105, 1998. DOI: 10.1073/pnas.95.21.12100

17. Liu G, Xiong YL: Contribution of lipid and protein oxidation to rheological differences between chicken white and red muscle mvofibrilla proteins. *J Agric Food Chem*, 44, 779-784, 1996. DOI: 10.1021/jf9506242

18. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA*, 87 (4): 1620-1624, 1990. DOI: 10.1073/pnas.87.4.1620

19. Hu P, Luo X, Du F: A review on purification about calpains of bovine m. longissimus by deae-sepharose-ff ion-exchange chromatography. *Innovational Edition of Farm Products Processing*, 59, 43-45, 2010.

20. Brooks JC, Savell JW: Perimysium thickness as an indicator of beef tenderness. *Meat Sci,* 67, 329-334, 2004. DOI: 10.1016/j.meatsci.2003.10.019

21. Light N, Champion AE, Voyle C, Bailey AJ: The role of epimysial, perimysial and endomysial collagen in determining texture in six bovine muscles. *Meat Sci*, 13 (3): 137-149, 1985. DOI: 10.1016/0309-1740(85)90054-3

22. Veiseth E, Shackelford SD, Wheeler TL, Koohmaraie M: Indicators of tenderization are detectable by 12 h postmortem in ovine longissimus. *J Anim Sci*, 82 (5): 1428-1436, 2004. DOI: 10.2527/2004.8251428x

23. Kolczak T, Pospiech E, Palka K, Łącki J: Changes in structure of *psoasmajor* and *minor* and *semitendinosus* muscles from calves, heifers and cows during postmortem aging. *Meat Sci*, 64, 77-83, 2003. DOI: 10.1016/S0309-1740(02)00164-X

24. Bo X, Guanghong Z, Xinglin X, Chunbao L: Venison in different parts of the maturation process after slaughter ultrastructure. *Bulletin of Nanjing Agricultural University*, 31 (1): 107-111, 2008.

25. Hopkins DL, Thompson JM: Inhibition of protease activity. Part 1. The effect on tenderness and indicators of proteolysis in ovine muscle. *Meat Sci*, 59 (2): 175-185, 2001. DOI: 10.1016/S0309-1740(01)00068-7

26. Koohmaraie M: Quantification of Ca^{2+}-dependent protease activities byhydrophobic ion-exchange chromatography. *J Anim Sci*, 68, 659-665, 1990. DOI: 10.2527/1990.683659x

27. Bhat ZF, Morton JD, Mason SL, Bekhit AEA: Role of calpain system in meat tenderness: A review. *Food Sci Hum Well*, 7, 196-204, 2018. DOI: 10.1016/j.fshw.2018.08.002

28. Kristensen L, Christensen M, Ertbjerg P: Activities of calpastatin, μ-calpain and m-calpain are stable during frozen storage of meat. *Meat Sci*, 72 (1): 116-120, 2006. DOI: 10.1016/j.meatsci.2005.06.010

29. Kristensen L, Therkildsen M, Riis B, Sørensen MT, Oksbjerg N, Purslow PP, Ertbjerg P: Dietary-induced changes of muscle growth rate in pigs: Effects on *in vivo* and postmortem muscle proteolysis and meat quality. *J Anim Sci*, 80 (11): 2862-2871, 2002. DOI: 10.2527/2002.80112862x

30. Whipple G, Koohmaraie M: Calcium chloride marination effects on beef steak tenderness and calpainproteolytic activity. *Meat Sci*, 33 (2): 265-275, 1993. DOI: 10.1016/0309-1740(93)90064-O

31. Pringle TD, Harrelson JM, West RL,Williams SE, Johnson DD: Calcium-activated tenderization of strip loin, top sirloin, and top round steaks in diverse genotypes of cattle. *J Anim Sci*, 77 (12): 3230-3237, 1999. DOI: 10.2527/1999.77123230x

32. Cheng LI, Xie T, Gang FU, Dai-wen C: Correlation analysis among tenderness indexes during postmortem chilling aging of pork. *Food Sci*, 30 (17): 163-166, 2009.

33. Telci A, Çakatay U, Salman S, Satman I, Sivas A: Oxidative protein damage in early stage Type 1 diabetic patients. *Diabet Res Clin Pract*, 50 (3): 213-223, 2000. DOI: 10.1016/s0168-8227(00)00197-2

34. Grune T, Blasig IE, Sitte N, Roloff B, Haseloff R, Davies KJ: Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J Biol Chem*, 273 (18): 10857-10862, 1998.

35. Rowe LJ, Maddock KR, Lonergan SM, Huff-Lonergan E: Oxidative environments decrease tenderization of beef steaks through inactivation of μ-calpain. *J Anim Sci*, 82 (11): 3254-3266, 2004. DOI: 10.2527/2004.82113254x

Kafkas Univ Vet Fak Derg
26 (2): 247-254, 2020
DOI: 10.9775/kvfd.2019.22856

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Evaluation of Quality Parameters of Chicken Eggs Stored at Different Temperatures

Sema SANDIKÇI ALTUNATMAZ [1,a] Filiz AKSU [1,b] Deniz AKTARAN BALA [1,c]
İbrahim AKYAZI [2,d] Cansu ÇELİK [1,e]

[1] Istanbul University-Cerrahpasa, Vocational School of Veterinary Medicine, Food Processing Department, Food Technology Programme, TR-34320, Avcilar/Istanbul - TURKEY
[2] Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Physiology, TR-34320, Avcilar/Istanbul - TURKEY
[a] ORCID: 0000-0001-9437-3366; [b] ORCID: 0000-0003-0532-4293; [c] ORCID: 0000-0003-1512-8552; [d] ORCID: 0000-0002-8808-8216
[e] ORCID: 0000-0002-9508-7473

Article ID: KVFD-2019-22856 Received: 20.06.2019 Accepted: 29.11.2019 Published Online: 30.11.2019

How to Cite This Article

Sandıkçı Altunatmaz S, Aksu F, Aktaran Bala D, Akyazı İ, Çelik C: Evaluation of quality parameters of chicken eggs stored at different temperatures. *Kafkas Univ Vet Fak Derg,* 26 (2): 247-254, 2020. DOI: 10.9775/kvfd.2019.22856

Abstract

This study was carried out to evaluate the effect of temperature and time on the internal and external quality of chicken eggs stored at room and fridge temperature for 28 days. A total of 176 eggs of Lohmann Brown (LSL) laying hens were used for the study. The eggs from the same batch were stored at fridge (5°C) and room temperature (25°C), internal and external quality parameters and microbiological analyzes of eggs were evaluated on days 0, 1, 8, 18, 21 and 28. No coliform bacteria, *E. coli* and *Salmonella* spp. were detected. At the end of the evaluation, the eggs stored at room temperature were found to have been negatively affected in terms of albumen index and Haugh unit parameters from day 8 onwards. When a comparison was made between the eggs kept in the room and the fridge temperature, significant differences were found in terms of albumen index, Haugh unit, egg yolk index and egg yolk height parameters ($P<0.05$). As a result, it is predicted that the storage temperature and duration play an important role in preserving the freshness of the eggs and the eggs should be kept under the cold chain, especially from day 8 onwards after the egg laying date.

Keywords: *Chicken egg, Storage temperature, Storage duration, Shelf life, Quality*

Farklı Sıcaklıklarda Depolanan Tavuk Yumurtalarının Kalite Parametrelerinin Değerlendirilmesi

Öz

Bu araştırma oda ve buzdolabı sıcaklığında 28 gün depolanan tavuk yumurtalarında sıcaklık ve sürenin yumurtaların iç ve dış kalitesine etkisini değerlendirmek amacıyla yapıldı. Araştırma için Lohmann Brown (LSL) cinsi yumurtacı tavuklara ait toplam 176 adet yumurta kullanıldı. Aynı partiden alınan yumurtalar buzdolabı (5°C) ve oda sıcaklığında (25°C) muhafaza edildi. 0, 1, 8, 18, 21 ve 28. günlerde, yumurtaların iç ve dış kalite parametreleri ile mikrobiyolojik analizleri değerlendirildi. Örneklerin hiçbirinde koliform bakteri, *E. coli* ve *Salmonella* spp. tespit edilmedi. Değerlendirme sonucunda, oda sıcaklığında muhafaza edilen yumurtalar 8. günden itibaren yumurta akı indeksi ve Haugh ünitesi parametreleri açısından belirgin düzeyde olumsuz etkilendi. Oda ve buzdolabı sıcaklığında muhafaza edilen yumurtalar arasında kıyaslama yapıldığında; albumen indeksi, Haugh ünitesi, yumurta sarısı indeksi ve yumurta sarısı yüksekliği parametreleri açısından anlamlı farklar olduğu belirlendi ($P<0.05$). Sonuç olarak, yumurta tazeliğinin korunmasında depolama sıcaklığının ve sürenin önemli bir rolü olduğu, yumurtaların yumurtlama tarihinden sonra en fazla 8. günden itibaren soğuk zincirde muhafaza edilmesi gerekliliği öngörülmüştür.

Anahtar sözcükler: *Tavuk yumurtası, Depolama sıcaklığı, Depolama süresi, Raf ömrü, Kalite*

INTRODUCTION

Egg is an important animal food that contains many nutrients required for a balanced human nutrition. In particular, it is indispensable for being rich in essential amino acids, its digestibility of 95% and its biological value of 100% [1-3].

All eggs offered in retail markets as well as used in the production processes as a raw material must be fresh, reliable and meet the quality criteria. The eggs can be exposed to microbial contamination during storage, transportation, sales and also quality losses depending on the storage conditions. Egg quality is significantly influenced by

İletişim (Correspondence)
+90 212 4737070/17153
deniz.bala@istanbul.edu.tr

environmental conditions such as temperature, humidity and also duration of storage [4]. Various studies were carried out to minimize the quality changes in eggs. In the long-term preservation of eggs, some technologies such as cold storage, UV, ozone, modified atmosphere packaging, coating and washing are used [5,6].

The eggshell is covered with a layer of water-proof mucous featured cuticle, which is very thin (5-10 µm). The cuticle layer generates a barrier for microorganisms and a natural defence mechanism for the egg while allowing the passage of gas and moisture. This layer may lose its properties in a short time after the egg laying. The cuticle layer can be destroyed via mechanical washing of the eggs and thus the mechanism of preventing the eggs from microorganism penetration may be impaired [6-8]. There are about 7500 pores in the eggshell. Depending on the storage conditions, the size of these pores increases and allows microorganisms to penetrate the egg [9].

It is possible to cover the eggs with various coating materials and to extend the storage period [10]. According to the egg relevant legislation in Turkey, an A-class egg cannot be cleaned by washing or any other cleaning method and must not be oiled. Therefore, the best preservation method for the eggs sold in retail markets in order to limit the quality changes caused by the storage is the cold storage. It is stated that the best conditions for the cold storage are 4-5°C and moisture of 75-85% [11].

The storage of the egg in improper storage conditions causes a change in the quality parameters within a few days. Especially the air sac widths, albumen and egg yolk height are affected by these changes.

Changes in egg albumen are the most prominent parameters that vary depending on storage temperature and duration. Depending on the storage duration and temperature, the height of the egg albumen which is measured from the nearest part of egg yolk, reduces, also thins and the fluidity increases. Depending on the decrease in density, the fluidity towards the thin albumen section increases. Increased pH and loss of carbon dioxide depending on the progress of the storage process causes changes especially in lysozyme-ovomucine complex as an egg white protein. This has negative effects on the gelatinous structure of the albumen. Another important alteration of the storage process having an effect on egg quality is a decrease in egg yolk height and the easy disintegration of the storage in the subsequent processes depending on the thinning of vitelline membrane. Another change is regarding pH values. The pH of egg yolk is approximately 6.0 and does not contain carbon dioxide. Depending on the storage process the egg yolk pH value does not show any significant change. The pH value of albumen is initially about 7.6. Depending on storage time, due to the outflow of carbon dioxide, pH can rise to about 9.0 [4,8,12].

Changes in egg quality parameters affect consumers' consumption and quality classification of the egg. The eggshells of high quality, class-A eggs sold in retail markets in Turkey must be clean, uncracked, unbroken and have a normal shape. Air sac cannot be higher than 4 mm in eggs for eggs sold as "extra fresh" (from the date of egg laying up to the ninth day) and must not exceed 6 mm for others. Egg albumen should be clear, transparent, gel and free from foreign substances. Egg yolk should be in the center of the egg and free from foreign substances. The egg should not contain a visible embryo and should not contain foreign odor. According to the egg-related Turkish legislations, there is no obligation for eggs to be cooled until the 18th day after the egg laying, however, they must be stored between (+8)/(+5)°C after day 18. Under these conditions, it is stated that a class A egg should be delivered to the consumer within 21 days from the laying date and the expiry date must be within 28 days from the date of egg laying [11]. One of the most important quality parameters for the egg is the Haugh unit value. This value varies considerably with the changes in the albumen of the egg depending on the storage. The Haugh unit value can show variations in direct proportion to the egg weight. Turkish Food Codex does not specify any parameters for this value.

It is possible to slow down the spoilage and undesired quality changes when the eggs are stored in appropriate storage conditions from the time they are taken from the chicken. Therefore, the purpose of this study is to evaluate the effects of the preservation of the eggs at room and fridge temperatures on some quality parameters.

MATERIAL and METHODS

Clean, brown, unfertilized, fresh (from day 0) and uncleared eggs were used in this research. A total of 176 eggs of Lohmann Brown (LSL) chickens were used for the study. The eggs were randomly selected from those chickens which had laid eggs on the same day. Egg samples were delivered to the laboratory within 2 h complying with the aseptic conditions. Selected eggs were divided into two equal groups. The first group, the ambient temperature group (AT) was stored in a 25°C incubator (Nüve) whereas the other group, the fridge temperature group (FT) was stored in a 5°C fridge (Siemens). The day-0 eggs were analysed. Each of the AT and FT groups included 5 eggs. The internal and external quality parameters of the eggs were measured on days 0, 1, 8, 18, 21 and 28. The research was conducted in two repetitions between June 2018 and August 2018. The average of the measurement results was evaluated statistically.

Thirteen different parameters were measured to evaluate the quality changes of the eggs depending on their storage.

Internal Quality Policy: Albumen index, Haugh unit, albumen height, egg yolk index, albumen ratio, egg yolk ratio, egg yolk height, albumen weight and egg yolk weight.

External Quality Parameters: Egg shape index, eggshell

percentage, egg weight and eggshell thickness. After eggs were collected and brought to the laboratory, they were grouped and weighed with their shells before storage. During the storage period, the weight of the eggs were measured and the width and height diameters were also measured by a digital caliper. The eggs were broken in a clean petri dish. The diameter and height of the egg yolk (from the center of the yolk), albumen height (from the side of the chalaza region), albumen diameter and width were measured by a caliper. Then, egg yolk and albumen were separated and each was weighed on a sensitive scale (Radwag).

Eggshells were also transferred to a clean petri dish and were weighed. Then, the eggshells were left to dry in an incubator at 37°C for 24 h. The eggshells were measured from two different parts (side and top) after they had been dried in the incubator. The pH values of egg yolk and albumen were measured by pH meter (Hanna HI 2211).

All parameter measurements were made for all eggs on determined days. The data obtained from the measurements were used to determine the following parameters: [13-18].

Albumen index: Albumen height/(Albumen length + Average of albumen width) *100

Albumen ratio: Albumen weight/Egg weight * 100

Egg yolk index: Egg yolk height/Egg yolk diameter * 100

Egg yolk ratio: Egg yolk weight/Egg weight * 100

Eggshell ratio: Eggshell weight/Egg weight * 100

Haugh unit: 100 log (Albumen height-1.7* Egg weight $^{0.37}$+7.6)

Egg shape index: Egg width/Egg length * 100

Eggshell percentage: Eggshell thickness/Egg weight * 100

Microbiological analyses of eggs were performed from day 0 onwards and during the storage conditions. The analyses were carried out separately for inner and shell part of the eggs allocated for microbiological analyses at specified times. Total mesophilic aerobic bacteria [19], *Salmonella* spp. [20], coliform bacteria [21], *E. coli* [22] and mold-yeast [23] were analyzed for both inner and shell area of the egg.

The eggshell was disinfected with 70% of alcohol to analyze the inside of the egg. Then, the egg was broken under aseptic conditions and analysed. For the analyses of the shell area of the egg, the eggs were washed with the dilution of a 1:9 physiological saline. Then, total mesophilic aerobic bacteria, coliform bacteria, *E. coli* and mold-yeast microbiological analyses were carried out.

Shell eggs were incubated inside buffered peptoned water in order to determine *Salmonella* spp. and for further continued analysis.

Statistical Evaluation: The data of all groups were tested for normality via Shapiro-Wilk tests. Data found to be normally distributed were then analyzed with one-way ANOVA. The non-parametric Kruskal-Wallis tests were conducted for data not normally distributed. The Mann-Whitney U tests with Bonferroni correction were used for pairwise comparisons between groups. Differences at P=0.05 level were accepted as being statistically significant. SPSS software was used for statistical analysis (SPSS for Windows, edition 17.0 (Release 17.0.0 - Aug 23, 2008).

RESULTS

The results obtained from the research we conducted in order to evaluate the quality parameters in the chicken eggs stored at different storage temperature are shown in *Table 1, 2, 3* and *Fig. 1, 2, 3, 4*. According to these results, it is observed that there are significant changes in the internal quality characteristics of the eggs due to the temperature differences during the storage period. The changes in the external quality characteristics are not statistically significant.

When the microbiological analysis findings were evaluated, there was no coliform bacteria. *E. coli* and *Salmonella* spp. detected in any of the interior and exterior areas of the egg samples.

On day 0, in eggs' internal area microbiological analyses, total mesophilic aerobic bacteria were detected with the number of 5x10^2 CFU/mL. In addition, the high yeast presence was remarkable. In eggs' external area microbiological analyses, total mesophilic aerobic bacteria were detected with the number of 1.2x10^3 CFU/mL only from the fridged storage eggs analysed on day 18. Except for these findings, there was no microbial growth or it was the detected to be below limits.

In our study, there was no significant difference in pH change between groups. The pH changes in albumen and egg yolk dependent on the time and temperature are shown in *Table 3*.

DISCUSSION

In this study, the groups kept at room (25°C) and fridge (5°C) temperature were evaluated within and between the groups in terms of internal and external quality criteria. While temperature and time did not significantly affect the external quality characteristics of the eggs (P>0.05), it was found that it remarkebly affected most of the internal quality characteristics (P<0.05) *(Table 1, 2)*. In our study, the internal quality characteristics of the eggs were adversely affected by being kept at room temperature, compared to the eggs kept at the fridge temperature. Differences in albumen index, egg yolk index, HU and egg yolk height were found to be statistically significant when the two groups were compared (P<0.05).

Although weight loss occurred in the eggs in both groups of our study, this change was not found to be statistically

Table 1. *Quality changes in eggs stored at fridge temperature*

Group	Albumen Index (%)		Egg White Height (mm)		Haugh Unit		Egg Shape Index (%)		Egg Yolk Index (%)		Egg White Ratio (%)		Egg Yolk Ratio (%)		Eggshell Percentage (%)		Egg Weight (g)		Egg Yolk Height (mm)		Egg White Weight (g)		Egg Yolk Weight (g)		Eggshell Thickness (mm)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FT1	3.63	0.85	6.71[b]	0.386	80.95	1.61	79.35	0.7	48.41	1.18	61.47	3	22.7	1.3	9.6	0.34	55	2.09	17.35[a]	0.45	33.63	1.68	12.42	0.709	0.32	0.03
FT8	4.36	0.62	8.27[a]	0.22	91.66	1.03	78.27	0.83	44.29	0.4	60.68	1.19	23	0.9	9.4	0.44	53.6	1.38	16.11[a]	0.23	32.63	1.42	12.28	0.33	0.31	0.01
FT18	3.76	0.16	7.44[ab]	0.12	85.7	1.74	79.1	0.92	41.75	0.98	56.19	1.06	24	0.4	9.1	0.34	53	0.78	15.52[ab]	0.27	29.82	0.94	12.74	0.18	0.29	0.02
FT21	3.18	0.68	6.75[b]	0.21	85.41	3.67	79.2	1.08	43.13	0.75	57.29	1.19	24.6	0.5	9.2	0.16	52.6	1.51	16.00[ab]	0.38	30.23	1.44	12.91	0.35	0.24	0.02
FT28	3.67	0.33	6.54[b]	0.26	79.48	2.38	80.89	0.9	40.06	0.62	53.35	1.64	23.9	1.5	9.4	0.39	53.1	1.76	14.87[b]	0.23	28.38	1.2	12.6	0.58	0.38	0.04
P -value	>0.05		<0.05		>0.05		>0.05		>0.05		>0.05		>0.05		>0.05		>0.05		<0.05		>0.05		>0.05		>0.05	

[a,b] *There is a significant differences between the means indicated with different letters on the same column (P<0.05)*

Table 2: *Quality changes in eggs stored at room temperature*

Group	Albumen Index (%)		Egg White Height (mm)		Haugh Unit		Egg Shape Index (%)		Egg Yolk Index (%)		Egg White Ratio (%)		Egg Yolk Ratio (%)		Eggshell Percentage (%)		Egg Weight (g)		Egg Yolk Height (mm)		Egg White Weight (g)		Egg Yolk Weight (g)		Eggshell Thickness (mm)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
RT0	4.92[a]	1.65	9.20[a]	1	89.94[a]	6.87	77.95	0.51	48.54[a]	1.93	60.2	1.58	21.2	0.75	9.25	0.37	54.77	1.46	17.14[a]	0.4	33	1.55	11.66	0.57	0.36	0.06
RT1	3.99[a]	0.47	8.39[ab]	0.52	92.38[a]	3.93	79.76	1.14	49.39[a]	0.82	59.2	1.52	23.7	0.71	10.5	1.53	54.55	0.28	17.56[a]	0.1	32.4	0.93	12.91	0.36	0.27	0.03
RT8	2.34[a]	0.12	5.58[b]	0.62	73.52[a]	6.8	84.96	5.22	37.88[b]	1.63	59.2	0.63	23.6	0.72	9.91	0.14	52.76	1.48	14.12[b]	0.5	31.3	0.98	12.44	0.63	0.27	0.01
RT18	0[b]		0[c]		0[b]		78.69	0.96	27.77[c]	0.83	54.6	1.11	26.2	1.34	9.92	0.2	53.45	1.55	11.40[c]	0.4	29.2	1.12	13.93	0.65	0.29	0.01
RT21	0[b]		0[c]		0[b]		79.68	0.08	28.81[c]	1.04	55.9	1.5	26.7	1.4	9.43	0.28	53.19	1.36	11.40[c]	0.4	29.8	1.48	14.14	0.54	0.28	0.02
RT28	0[b]		0[c]		0[b]		78.44	1.27	23.32[c]	1.59	55.4	1.98	26.1	1.4	9.06	0.3	53.48	2.08	9.98[c]	0.6	29.6	1.21	13.71	0.64	0.4	0.03
P -value	<0.05		<0.05		<0.05		>0.05		<0.05		>0.05		>0.05		>0.05		>0.05		<0.05		>0.05		>0.05		>0.05	

[a,b] *There is a significant differences between the means indicated with different letters on the same column (P<0.05)*

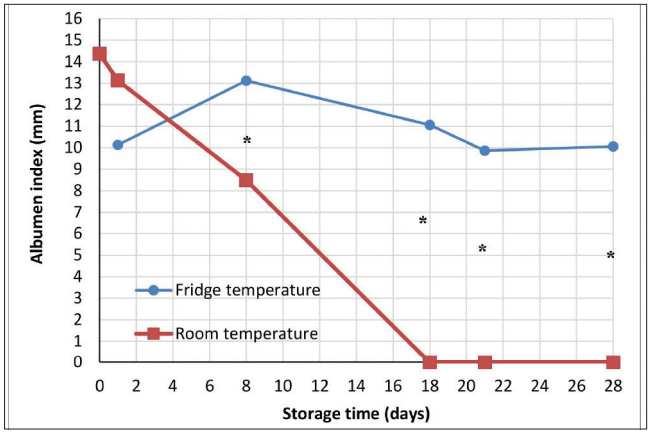


Fig 1. Effect of storage time and temperature on albumen index (mm) of chicken eggs; * The difference between the marked results is significant

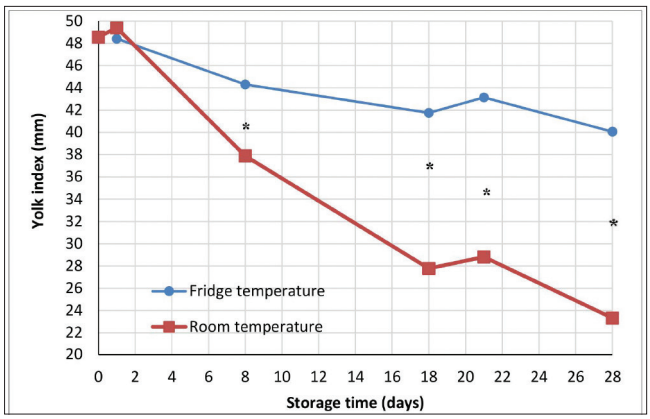


Fig 2. Effect of storage time and temperature on yolk index (mm) of chicken eggs; * The difference between the marked results is significant

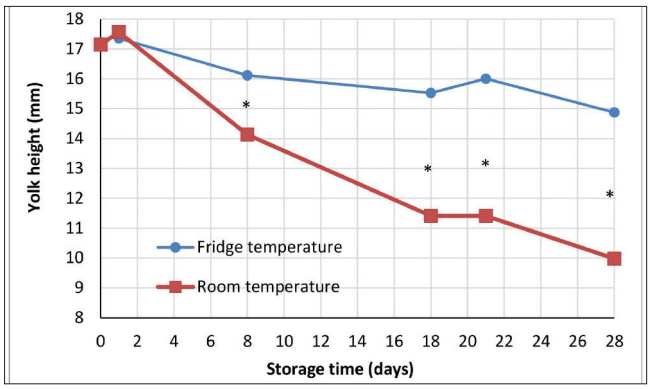


Fig 3. Effect of storage time and temperature on yolk height (mm) of chicken eggs; * The difference between the marked results is significant

significant (P>0.05). Several studies have been conducted to investigate the effect of storage time and temperature on the weight loss and internal quality characteristics of eggs. Eggs' weight loss depending on the storage time and the temperature was found to be significant by various researchers [3-5,7,12,24-26]. Şamlı et al.[26] stated that the cold storage did not affect the weight loss. Scott et al.[27] found that the weight loss in the eggs was not important depending on the storage in their research.

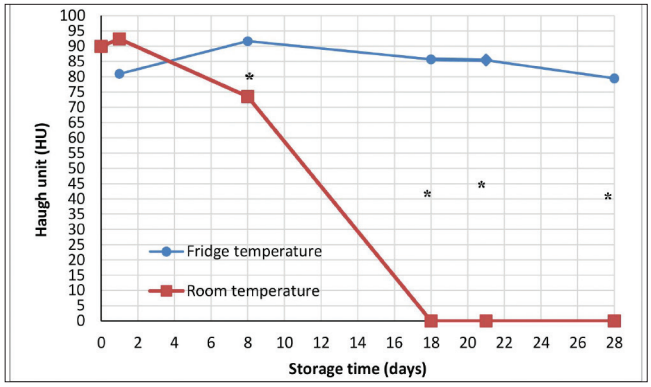


Fig 4. Effect of storage time and temperature on haugh unit (HU) of chicken eggs; * The difference between the marked results is significant

Table 3. *The pH changes in albumen and egg yolk dependent on the time and temperature*

Ambient Temperature	Day	Albumen	Egg Yolk
Room Temperature	Day 0	8.09	6.07
	Day 1	7.80	6.40
	Day 8	8.46	6.28
	Day 18	8.77	6.57
	Day 21	8.68	6.57
	Day 28	8.83	6.84
Fridge Temperature	Day 1	8.03	6.29
	Day 8	7.33	6.34
	Day 18	8.66	6.4
	Day 21	8.71	6.69
	Day 28	8.72	6.43

In our study, the mean average egg weight in the room temperature group decreased from 54.77 g to 53.48 g at the end of the day 28. The reason of the decrease in weight loss may be related with the ambient conditions. In our study, egg yolk weight increased in both storage temperature treatments. This value increase is not found to be statistically significant. The increase in egg yolk weight may have been caused by the diffusion of water from the albumen to the egg yolk depending on the extended storage time.

When the groups from both of the storage temperature treatments were compared with each other depending on the time, we found that there were statistically significant changes in albumen index, haugh unit, egg yolk index and egg yolk height (P<0.05). When the group stored at the fridge temperature was evaluated in terms of time, statistically significant results were only found for albumen height and egg yolk height parameters (P<0.05). When the other group stored at room temperature was evaluated in terms of time, albumen height, Haugh unit, egg yolk index and egg yolk height parameters were found to be statistically significant (P<0.05). In eggs stored at room

temperature, the albumen height, HU and albumen index values could not be calculated due to the inability of measuring some parameters after day 8. When the eggs kept in the fridge except those from day 8 group were analyzed, the albumen height did not show any significant change between the 1st and 28th days. The HU values of the eggs kept in the fridge were found in the fresh egg (Class A) values even on the 28th day. When the albumen of room temperature eggs were compared with the eggs kept at the fridge temperature, the values of the fridge group on the 28th day were found to be higher than the values of the room temperature eggs from the 8th day. Similar to our study, some researchers found that the storage temperature negatively affects the albumen index, egg yolk index, egg yolk height and albumen height [4-6,24].

Haugh unit value is one of the most important quality parameters which changes depending on the storage process of the egg. Changes in the value of haugh unit were investigated by various researchers. Jones and Musgrove [28] found that HU value decreased from 82.59 to 67.43 in 10 weeks and in cold conditions (4°C). Similarly, Jones et al.[29] also found that the HU value in eggs kept in cold storage for 12 weeks decreased from 84.62 to 66.21. Caner et al.[7] stated that HU value decreases within time and at the end of 5 weeks, it decreased from 81.23 to 58.93 in their research where the eggs were kept at 24°C for six weeks. In the study of Feddern et al.[30] the HU value decreased from 95.75 to 88.19 in the eggs they kept in the cold chain (0-5°C) for 3 weeks, whereas in the warm environments (20-30°C) the values were reduced from 98.60 to 51.92. According to our research results, HU values were found to be statistically significant between the groups kept at fridge versus at room temperature ($P<0.05$). The HU value in the group stored at the fridge temperature dropped to 79.48 from the initial value of 80.95 in 28 days. The changes in this period are not statistically significant in the fridge group ($P>0.05$). In the group stored at room temperature, the HU value decreased from 89.94 to 73.52 at the end of the 8th day. However, the HU parameter value could not be calculated since the quality of albumen was very low in our measurements since the 18th day. The HU value on the 28th day of the eggs stored in the fridge was found to be higher than the values on the 8th day of the group kept at room temperature. The preservation of eggs in the cold chain positively affects the quality of the HU value.

Albumen height is another important parameter in determining egg freshness. This parameter is also a critical value in the calculation of albumen index and HU values. In our study, the room temperature group's albumen height was initially 9.20 mm and then was calculated as 5.58 mm on the 8th day. In the room temperature group, no measurement could be obtained in the albumen height parameter from day 18. Even at the end of the 28th day in the fridge conditions, albumen height was measured and determined as 6.54 mm. Jones and Musgrove [28] found that albumen height in eggs were 7.05 mm on day 0 and 4.85 mm at the end of 10th week. Whereas Jones et al.[29] detected that the height of albumen falls from 7.21 mm to 4.80 mm after a 12 weeks of cold storage conditions. Şamlı et al.[26] found that the values of albumen height decreased from 8.56 mm to 6.18 mm (5°C) and 2.81 mm (29°C) after storing the eggs for 10 days (5, 21, 29°C). We observed that the albumen height values of the groups kept in the fridge were compatible with the results of the other researchers, however, the fact that we could not get the measurement from the room temperature group due to the increased albumen fluidity starting from day 18, limits our ability to compare.

As the albumen index parameter is a parameter associated with albumen height, the quality-related changes also affect these values. In our study, albumen index values are statistically significant between room temperature and fridge temperature groups ($P<0.05$). The fridge temperature was not found to be statistically significant when evaluated within the group ($P>0.05$) *(Table 1)*. In the group kept at room temperature, the albumen index value was statistically significant depending on the duration ($P<0.05$). However, albumen index values could not be detected after day 18 in this group. The reason for this is that albumen height could not be obtained due to increased albumen fluidity. In the research conducted by Artan and Durmus [31], it was found that the albumen index value was 4.94-5.87. These values are consistent with the findings of our study.

When egg yolk index parameter was evaluated, the egg yolk index values of the eggs at room temperature decreased significantly since 8th day, but the decrease in those kept at the fridge temperature was at a more reasonable level. When the egg yolk index results belonging to the day 28 were compared, it was seen that the egg yolk index of the eggs kept at the fridge temperature was in a better condition than the eggs kept at room temperature. The changes in egg yolk index were not statistically significant in the eggs stored in the fridge, but were found to be statistically significant in the eggs kept at room temperature ($P<0.05$). Şamlı et al.[26] study determined that the egg yolk index was 44.09 in fresh eggs; 40.77 (5°C) and 32.73 (29°C) at the end of 10 days. In our study, it was seen that the value of fridge group egg yolk index obtained from day 28 was compatible with the value taken from Şamlı et al.'s [30] fridge group (5°C) on 10th day.

In our study, there were significant differences between the two groups in terms of egg yolk height values ($P<0.05$). Egg yolk height decreased to 9.98 mm on the 28th day of the eggs kept at room temperature and 14.87 mm on the 28th day of the eggs kept at fridge temperature. It was determined that the 28th day value of the egg yolk height of the eggs that were kept in the fridge and the 8th day value of the eggs kept at room temperature were close to each other.

In our study, there were no statistically significant differences between the eggs stored in the room and fridge temperature

in terms of egg shape index, albumen ratio, egg yolk ratio, eggshell percentage, egg weight, albumen weight, egg yolk weight, eggshell thickness parameters (P>0.05).

The other parameter of the egg that can show changes due to storage is the pH value. In our study, there was no significant difference in pH between groups. Current differences are thought to be caused by individual differences in eggs. The pH of albumen in fresh eggs is between 7.55-8.5 [8]. In our study, pH values varied in the range of 7.52 to 8.90 in the eggs kept at room temperature while it varied in the range of 7.55 to 8.50 in the eggs kept in the fridge. Giampietro-Ganeco et al.[32] examined the quality differences of the eggs stored in the inner shelf and domestic fridge door and it was found that the quality characteristics of the eggs stored in the inner shelf were better protected. The same researchers stated that the pH of egg yolk and egg white did not show any significant change in this study. Şamlı et al.[26] specified that the pH of the albümin, which was initially recorded as 7.47, increased to 8.26 at 5°C and to 9.11 at 29°C degrees after 10 days. However, they added that the changes in egg yolk pH did not differ as much as the changes in the albumen. In Caner et al.[7] research where eggs were kept for 6 weeks at 24°C, the pH of the albumen in the first three weeks increased from 7.5 to 9.27 and the egg yolk pH increased from 5.86 to 6.32. Akyürek and Okur [4] stated that time (14 days) and temperature (4 and 22°C) significantly increased albumen and egg yolk pH.

In the microbiological investigations of inside and outside of the eggs, no coliform bacteria, *E. coli* and *Salmonella* spp. were detected in any of the samples. Total aerobic mesophilic bacteria and mold yeasts were detected in some eggs. Microbiological analyses are considered to be valuable in terms of giving some first insights only, rather than providing definite results results due to the individual differences of the analyzed eggs. Although the same conditions were provided, the fact that the same egg could not be used in the next analysis caused inconsistency between the results. Therefore, the analyzes were evaluated on one egg. At maximum, a total of 600 cfu (colony forming units) of total mesophilic aerobic bacteria and intense yeast were found in the interior of some eggs. The total number of mesophilic aerobic bacteria that can be obtained at maximum level in the microbiological analysis of the eggs' outer area stored at both storage temperatures was 1200 cfu and also 144 cfu mold colonies have been detected at maximum level. Various researchers have conducted studies to determine the microbiological quality of eggs. In the study of Eke et al.[4] the total mesophilic aerobic bacteria level was found to be $5x10^3$ CFU/mL and the mold-yeast level was $9x10^2$ CFU/mL at the ambient temperature of week zero. They stored these eggs at ambient (32+2°C) and fridge temperature for four weeks. As a result of the analyses, they found a total of $2.8x10^7$ and $1.1x10^4$ CFU/mL total mesophilic aerobic bacteria respectively. They stated that they had detected $1.2x10^5$ CFU/mL mold-yeast at the end of the fourth week in the group they kept in ambient conditions (32°C). The reason why the numbers are considered to be particularly high is that the cuticle on the surface of the eggs stored at ambient temperature dried faster and began to shrink. This causes an increase in pore size and ease of the penetration of microorganisms into the eggshell. It was stated that the mold-yeast population in the eggs stored at ambient temperature may be due to the humidity condition of the medium. In the study of Park et al.[6] the groups were set by treating the eggs with mineral oil, washing and without any process, and then they were evaluated them microbiologically. The researchers determined the initial microflora in eggs (day 0. at 30°C) as $2.8x10^2$ CFU/mL. In our study, we obtained similar results from some eggs, but our results were generally below the detection limits.

The effects of temperature and duration are evaluated in most of the studies related to the quality change of the egg. The importance of temperature in sustainability of egg quality is very important. In the study of Yenilmez et al.[9] where they investigated the quality characteristics of the eggs kept in hot and cold conditions during summer and winter, it was stated that the values obtained from the eggs kept at 4°C were better. The researchers reported that the eggs kept in summer (33°C) can be stored safely for one week and those kept in 18°C can be stored for two weeks. Eke et al.[24] stated that HU, yolk index and pH values of eggs stored at 32°C are affected more than eggs kept under fridge conditions. Akter et al.[5] stated that in both fridge and room conditions egg weight loss, the percentage of egg yolk weight, egg yolk pH value and albumen pH value increased but Haugh units and the percentage of weight of albumen decreased. These researchers have stated that eggs maintain their quality for 28 days at room temperature and 14 days at room temperature. In the study conducted by Tabidi [12], it was stated that the eggs kept at 37°C lost their consumable properties on the 15^{th} day and the eggs kept their freshness at 4°C. In our study, for the eggs stored at room temperature, especially after the 18^{th} day, the albumen index, albumen height and Haugh unit values could not be obtained. As a result, it is predicted that the storage temperature and duration play an important role in maintaining the freshness of the eggs and also there is a necessity the eggs should be kept in the cold chain after 8 days from the date of laying. Lee et al.[33] stated that the storage temperature and duration are major factors affecting egg quality. When the storage temperature and duration are compared, it is emphasized that storage temperature is a more sensitive determinant. This finding supports the results of our study. Feddern et al.[30] stated that there was a rapid deterioration in the eggs stored at room temperature in 1 to 5 weeks, and recommended that these eggs should be consumed in 2 weeks to maintain their internal quality until they reach the consumer from the farm or stored in the refrigerator for up to 8 weeks.

In this study, the effects of the storage time and the

temperature on the quality parameters of the eggs kept at room and fridge temperature were evaluated. It was determined that preservation of the eggs in the cold environment was important for the quality criteria of the egg and that the ambient temperature adversely affected the egg quality in terms of freshness criteria. As a result, it is predicted that the storage temperature and duration play an important role in preserving the freshness of the eggs and the eggs should be kept under the cold chain. At the latest from day 8 onwards after the egg laying date. It is thought that this study will shed light on the studies for the preservation of eggs for a longer time, while preserving its quality characteristics.

Acknowledgment

We thank Pınar Ertör Akyazı for her assistance.

Conflict of Interest

There is no conflict of interest in the present study.

REFERENCES

1. Çelebi Ş, Karaca H: Egg nutritional value, cholesterol content and studies on the nutritionally enriched Egg with n-3 PUFAS. *Atatürk Üniv Ziraat Fak Derg,* 37 (2): 257-265, 2006.

2. Ruxton CHS, Derbyshire E, Gibson S: The nutritional properties and health benefit of eggs. *Nutr Food Sci,* 40 (3): 263-279, 2010. DOI: 10.1108/00346651011043961

3. De Menezes PC, de Lima ER, de Mederios JP, de Oliveira WNK, Evêncio-Neto J: Egg quality of laying hens in different conditions of storage, ages and housing densities. *Rev Bras Zootec*, 41 (9): 2064-2069, 2012. DOI: 10.1590/S1516-35982012000900014

4. Akyürek H, Okur AA: Effect of storage time, temperature and hen age on egg quality in free-range layer hens. *J Anim Vet Adv*, 8 (10): 1953-1958, 2009.

5. Akter Y, Kasim A, Omar H, Sazili AO: Effect of storage time and temperature on the quality characteristics of chicken eggs. *J Food Agric Environ,* 12 (3-4): 87-92, 2014.

6. Park YS, Yoo IJ, Jeon KH, Kim HK, Chang EJ, Oh HI: Effects of various eggshell treatments on the egg quality during storage. *Asian Australas J Anim Sci,* 16 (8): 1224-1229, 2003. DOI: 10.5713/ajas.2003.1224

7. Caner C, Yüceer M: Efficacy of various protein-based coating on enhancing the shelf life of fresh eggs during storage. *Poult Sci*, 94 (7): 1665-1677, 2015. DOI: 10.3382/ps/pev102

8. Anar Ş: Yumurta ve Yumurta Ürünleri. Yumurtanın Fiziksel Özellikleri. 30-35, Dora Yayınları, Bursa, 2016.

9. Yenilmez F, Uruk E, Serbester U, Çelik L: Farklı mevsimlerde toptancı ve tüketici koşullarında saklamanın yumurta kalitesi üzerine etkisi. Çukurova *J Agric Food Sci,* 32 (2): 61-68, 2017.

10. Al-Hajo NNA, Whaeep AAM, Rashid RS, Azeez AI, Al-Janab LFA, Musa TN, Al-Khalani FMHA: Effect of different coating material on egg quality. *Acad J Sci,* 1 (2): 257-264, 2012.

11. Turkish Food Codex Regulation on Egg Notification: Republic of Turkey Ministry of Agriculture and Forestry, Official Gazette Issue and Date: 29211 and 20 December 2014, Ankara, Turkey.

12. Tabidi MH: Impact of storage period and quality on composition of table egg. *Adv Environ Biol,* 5 (5): 856-861, 2011.

13. Reddy PM, Reddy VR, Reddy CV, Rao SP: Egg weight. shape index and hatchability in khaki Campbell duck egg. *Ind J Poult Sci,* 14, 26-31, 1979.

14. Doyon G, Bernier-Cardou M, Hamilton RMG, Castalgne F, Randall CJ: Egg quality 2. albumen quality of eggs from five commercial strains of white leghorn hens during one year of lay. *Poult Sci*, 65, 63-66, 1986. DOI: 10.3382/ps.0650063

15. Anderson KE, Tharrington JB, Curtis PA, Jones FT: Shell characteristics of eggs from historic strains of single comb white leghorn chickens and relationship of egg shape to shell strength. *Int J Poult Sci*, 3, 17-19, 2004. DOI: 10.3923/ijps.2004.17.19

16. Haugh RR: The Haugh unit for measuring egg quality. *US Egg Poult Mag*, 43, 572-573, 1937.

17. Chowdhury SD: Shell membrane system in relation to lathyrogen toxicity and copper deficiency. *World's Poult Sci J*, 46, 153-169, 1990. DOI: 10.1079/WPS19900018

18. Cayan H, Erener G: Effect of olive leaf *(Olea europaea)* Powder on laying hens performance, egg quality and egg yolk cholesterol levels. *Asian Australas J Anim Sci,* 28 (4): 538-543, 2015. DOI: 10.5713/ajas.14.0369

19. Maturin L, Peeler JT: Aerobic Plate Count. *Bacteriological Analytical Manual*, Chapter 3,2001.https://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm063346.htm.; *Accessed date:* 27/11/2019

20. ISO 6579: Microbiology of food and animal feeding stuffs-horizontal method for the detection of *Salmonella* spp. International Standard, 2002.

21. Erkan ME, Vural A, Güran HŞ: Diyarbakır ili'nde satışa sunulan köy ve market yumurtalarının hijyenik kalitesi üzerine bir araştırma. *Dicle Univ Vet Fak Derg*, 1 (1): 11-16, 2008.

22. ISO 16649-2: Microbiology of food and animal feeding stuffs - horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* - Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. 2001.

23. ISO 21527-1: Microbiology of food and animal feeding stuffs - horizontal method for the enumeration of yeasts and moulds - Part 1: Colony count technique in products with water activity greater than 0.95. ISO21527-1. 2008.

24. Eke MO, Olaitan NI, Ochefu JH: Effect of storage conditions on the quality attributes of shell (table) eggs. *Niger Food J*, 31 (2): 18-24, 2013. DOI: 10.1016/S0189-7241(15)30072-2

25. Bozkurt Z, Tekerli M: The effects of hen age, genotype, period and temperature of storage on egg quality. *Kafkas Unıv Vet Fak Derg,* 15 (4): 517-524, 2009. DOI: 10.9775/kvfd.2009.041A/Err

26. Şamlı HE, Ağma A, Şenköylü N: Effects of storage time and temperature on egg quality in old laying hens. *J Appl Poult Res,* 14 (3): 548-553, 2005. DOI: 10.1093/japr/14.3.548

27. Scott TA, Silversides FG: The effect of storage and strain of hen on egg quality. *Poult Sci*, 79 (12): 1725-1729, 2000. DOI: 10.1093/ps/79.12.1725

28. Jones DR, Musgrove MT: Effects of extended storage on egg quality factors. *Poult Sci*, 84 (11): 1774-1777, 2005. DOI: 10.1093/ps/84.11.1774

29. Jones DR, Karcher DM, Abdo Z: Effect of a commercial housing system on egg quality during extended storage. *Poult Sci*, 93 (5): 1282-1288, 2014. DOI: 10.3382/ps.2013-03631

30. Feddern V, de Prá MC, Mores R, da Silveira Nicoloso R, Coldebella A, de Abreu PG: Egg quality assessment at different storage conditions, seasons and laying hen strains. *Cienc Agrotec*, 41 (3): 322-333, 2017. DOI: 10.1590/1413-70542017413002317

31. Artan S, Durmuş I: Köy, serbest ve kafes sistemlerinde üretilen yumurtaların kalite özellikleri bakımından karşılaştırılması. *Akad Ziraat Derg*, 4 (2): 89-97, 2015.

32. Giampietro-Ganeco A, Scatolini-Silva AM, Borba H, Boiago MM, Lima TMA, Souza PA: Comparative study of quality characteristics of egg stored in domestic refrigerators. *Ars Vet,* 28 (2): 100-104, 2012.

33. Lee MH, Cho EJ, Choi ES, Sohn SH: The effect of storage period and temperature on egg quality in commercial eggs. *Korean J Poult Sci*, 43 (1): 31-38, 2016.

Kafkas Univ Vet Fak Derg
26 (2): 255-262, 2020
DOI: 10.9775/kvfd.2019.22908

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Genetic Polymorphism of *STAT1* and *STAT5A* Genes in Holstein, Jersey, and Indigenous Cattle Breeds in Turkey [1][2]

Ozden COBANOGLU [1,a] Ertugrul KUL [2,b] Samet Hasan ABACI [3,c]
Eser Kemal GURCAN [4,d] Soner CANKAYA [5,e]

[1] *The study was supported by TUBITAK with project number #110O821*
[2] *The study was presented at 8th International Balkan Animal Science Conference (BALNIMALCON 2017) in Prizren - Kosova, 6-8 September 2017*

[1] Department of Genetics, Faculty of Veterinary Medicine, University of Uludag, TR-16059 Bursa - TURKEY
[2] Department of Animal Science, Faculty of Agriculture, University of Kırşehir Ahi Evran, TR-40200 Kırşehir - TURKEY
[3] Department of Animal Science, Faculty of Agriculture, University of Ondokuz Mayis, TR-55139 Samsun - TURKEY
[4] Department of Animal Science, Faculty of Agriculture, University of Namik Kemal, TR-59030 Tekirdağ - TURKEY
[5] Department of Sport Management, Faculty of Yaşar Doğu Sport Sciences, University of Ondokuz Mayis, TR-55139 Samsun - TURKEY
[a] ORCID: 0000-0002-8247-9347; [b] ORCID: 0000-0003-4961-5607; [c] ORCID: 0000-0002-1341-4056; [d] ORCID: 0000-0002-9954-8126
[e] ORCID: 0000-0002-7002-1718

Article ID: KVFD-2019-22908 Received: 26.06.2019 Accepted: 255.10.2019 Published Online: 25.10.2019

How to Cite This Article

Cobanoglu O, Kul E, Abaci SH, Gurcan EK, Cankaya S: Genetic polymorphism of STAT1 and STAT5A genes in Holstein, Jersey, and indigenous cattle breeds in Turkey. *Kafkas Univ Vet Fak Derg,* 26 (2): 255-262, 2020. DOI: 10.9775/kvfd.2019.22908

Abstract

This study aimed to determine genetic polymorphism in *STAT1* and *STAT5A* genes for dairy cattle and some native cattle breeds in Turkey. 283 Jersey and a total of 472 Holstein cows from two different herds and 93 Grey Steppe, 85 Anatolian Black Cattle, and 66 East Anatolian Red cattle were used in this research. Generally, C allele gene frequency was higher than T allele for *STAT1* in all breeds whereas C allele gene frequency was detected higher than G allele for *STAT5A* in Jersey and East Anatolian Red. On the other hand, G allele gene frequency was higher than C allele in Holstein, Grey Steppe, and Anatolian Black Cattle breeds. The expected deviations from the Hardy-Weinberg Equilibrium were significant only for Jersey breeds for *STAT1* gene. Meanwhile, the expected deviation from equilibrium was also significantly different for Holstein in Black Sea Region (BSR), Anatolian Black Cattle and Grey Steppe for the *STAT5A* gene. FIS values were determined to *STAT1* gene as negative for all breeds except for Holstein in Marmara Region (MR). Similarly, this value was determined to *STAT5A* gene as positive for all breeds except for Holstein in BSR. The genetic distances for two loci were calculated between 0.0029 and 0.1599 among all populations. Depending on the cluster analysis, Holstein in BSR and MR, Anatolian Black Cattle, East Anatolian Red were closely clustered to each other, while Grey Steppe and Jersey were located in completely different clusters. As a conclusion, based on the detected genetic diversity in *STAT1* and *STAT5A* genes, it is possible to make a genetic improvement among bovine breeds raised in Turkey.

Keywords: *Cattle, Genetic polymorphism, Genetic relationships, STAT1, STAT5A*

Türkiye'de Holstein, Jersey ve Yerli Sığır Irklarında *STAT1* ve *STAT5A* Genlerinin Genetik Polimorfizmi

Öz

Bu çalışma, Türkiye'de süt sığırları ve bazı yerli sığır ırklarında *STAT1* ve *STAT5A* genlerine ait genetik polimorfizmin belirlenmesini amaçlamaktadır. Araştırma kapsamında, 283 Jersey ve iki farklı sürüden toplam 472 Siyah Alaca inekleri ile 93 Boz Irk, 85 Yerli Kara ve 66 Doğu Anadolu Kırmızısı sığırları kullanılmıştır. Genel olarak, tüm ırklarda C allel gen frekansı STAT1 için T allelinden daha yüksek olurken Jersey ve Doğu Anadolu Kırmızısı'n da ise C allel gen frekansı *STAT5A* için G allelinden daha yüksektir. Diğer yandan ise, G allel gen frekansı Siyah Alaca, Boz Irk ve Yerli Kara ırklarında C allelinden daha yüksektir. Hardy-Weinberg Dengesinden beklenen sapmalar sadece *STAT1* genin için Jersey ırkında önemlidir. Ayrıca, *STAT5A* geni için dengeden beklenen sapma Karadeniz Bölgesi'nde (KB) ki Siyah Alacalar, Yerli Kara ve Boz Irkları için de anlamlı derecede farklıdır. FIS değerleri, *STAT1* geni bakımından Marmara Bölgesi'nde (MB) ki Siyah Alacalar dışında bütün ırklar için negatif olarak belirlenmiştir. Benzer şekilde, bu değer, *STAT5A* geni bakımından KB'de ki Siyah Alacalar hariç bütün ırklar için pozitif olarak belirlenmiştir. İki lokusun genetik mesafeleri tüm popülasyonlar bakımından 0.0029 ile 0.1599 arasında hesaplanmıştır. Kümeleme analizine bağlı olarak, KB ve MB'de ki Siyah Alacalar, Yerli Kara, Doğu Anadolu Kırmızısı birbirlerine çok yakın kümelenmişken, Boz Irk ve Jersey ırkları tamamen farklı kümeler de yer almıştır. Sonuç olarak, *STAT1* ve *STAT5A* genlerinde tespit edilen genetik çeşitliliğe dayanarak Türkiye'de yetiştirilen büyükbaş hayvan ırkları arasında genetik bir iyileştirme yapılması mümkün görülmektedir.

Anahtar sözcükler: *Sığır, Genetik polimorfizm, Genetik ilişkiler, STAT1, STAT5A*

İletişim (Correspondence)
+90 532 0625770
ocobanoglu@uludag.edu.tr

INTRODUCTION

Determination of the genes with an effect on economically important yield traits in livestock species will provide faster progress in animal breeding as compared with the traditional selection practices. Besides knowing and preserving genetic diversity among farm animals, it is essential for the continuation of the existence of these species in the future and will also provide the implementation of right strategies [1]. Molecular genetic markers are widely used to achieve these purposes because molecular markers were reported to be more reliable in determining the genetic structure and widely used to discover the phylogenetic relationships among species as well as among breeds [2].

One of the essential genes that are thought to affect the yield traits of animals is the *STAT* gene family, which is known as Signal Transducers and Activators of Transcription Factors. This gene family has several different known forms as *STAT1*, *STAT2*, *STAT3*, *STAT4*, *STAT5*, and *STAT6* [3]. There are two isoforms of *STAT5* which are described as *A* and *B* form due to the difference of few amino acids at the carboxcylic end of protein molecule. Of this large gene family, the *STAT1* and *STAT5A* genes are frequently used in the candidate gene analyses. The bovine *STAT1* gene is located at 60 to 63 cM in chromosome 2 [4]. However, *STAT5A* maps to chromosome 19 containing 19 exons with 794 amino acid chains [5].

The different genomic regions discovered to be active on milk yield traits in the last decade were mapped in many dairy cattle breeds [6]. In many studies conducted, the relationship between the phenotypic traits and different alleles of the candidate gene in the population were investigated [7]. There are some evidence that *STAT1* plays an essential role in developmental process and a differentiation of the mammary gland [8,9]. Within this concept, *STAT1* gene was searched to determine the relationships between the genetic structure and yield traits of the herd in Czech Fleckvieh cattle. In that study, the frequencies of the *CC*, *CT,* and *TT* genotypes were as 71.60%, 26.75%, and 2.15%, respectively. Furthermore, significant differences in milk protein contents were observed among the all genotypes [10]. In a previous study performed for the *STAT1* gene, the effects of *CC* and *CT* genotypes calculated as a deviation from *TT* genotype had a significant impact of increasing milk yield as well as milk protein and fat yields in North American Holstein cows [11].

In a study where the relationships between meat yield traits and the *STAT5A* gene were investigated in Holstein cows reared in China, the frequencies of *CC*, *CT,* and *TT* genotypes were 0.79, 0.21, and 0.0 for this gene, respectively. It was reported that animals with *CC* genotype were more advantageous in terms of meat yield traits [12]. On the other hand, the *STAT5A* gene is also expressed to play a significant role in carrying signals, particularly from prolactin to milk protein genes [13]. The studies about *STAT5A* gene revealed that the significant effects on milk fat content and milk yield traits were observed in the Holstein cows reared in Poland and USA, respectively [14,15].

The effects of the *STAT5A* polymorphism on milk yield traits in Jersey cattle were also studied by Dario and Selvaggi [16]. They reported that *CC* genotype was expressed to be selectively advantageous in milk yield traits as compared to those with *CT* genotype. However, the herd was not in Hardy-Weinberg Genetic Equilibrium. On the contrary, the other study proved that animals carrying *TT* genotype for *STAT5A* gene turned out to be significantly different from those with *CT* genotype in term of milk fat content in Jersey cattle [17].

Although there are many studies conducted about the polymorphic effect of *STAT* gene family in many countries, there are not many studies conducted about these genes in cattle breeds raised in Turkey. Therefore, the purpose of this study was to precisely determine genetic polymorphism and genetic diversity of the *STAT1* and *STAT5A* genes in two different dairy breeds; as Holstein and Jersey cattle, and also in some indigenous cattle breeds; as Anatolian Black Cattle, East Anatolian Red, and Grey Steppe raised in Turkey.

MATERIAL and METHODS

Dairy and native cattle breeds were used as the animal material in this study. The dairy breeds were composed of 283 Jersey from TIGEM - Karakoy Agricultural Management in Bafra, Samsun, and 163 Holstein cattle from a commercial farm in Carsamba, Samsun at the Black Sea Region and 309 Holstein cattle from a commercial farm in Bursa at the Marmara Region. On the other hand, the native breeds were comprised of 93 Grey Steppe cattle from Bandirma Sheep Research Institute in Balıkesir and a commercial farm at Thrace Region, 85 Anatolian Black Cattle from Lalahan Livestock Central Research Institute in Ankara at Central Anatolia Region, and 66 East Anatolian Red cattle reared from breeders in Ardahan at Eastern Anatolia Region of Turkey. All the animals used in the study were chosen randomly from the herds.

Based on the study, blood samples from the jugular vein were collected into 10 mL vacuum tubes coated with K_2EDTA anticoagulant and DNA extractions were performed using a standard phenol/chloroform method [18]. Isolated DNA samples were used in PCR reaction for a technique of restriction fragment length polymorphism *(PCR-RFLP)*. The quality and quantity of DNA samples were evaluated using NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., USA).

In the amplification of genomic DNA, 50 ng of genomic DNA, 50 µM of each primer (forward and reverse), 200 µM of each dNTP, 2.5 µL of 10x PCR buffer solutions and 0.3 U (unit) of the *Taq* Polymerase were used in the PCR to have a total volume of 25 µL. According to the PCR protocol, the

Table 1. *Primer information for the STAT1 and STAT5A genes*

Gene	Primer Sequence (5′ to 3′)	Enzyme Digestion	PCR Product Size (bp)	Gen Bank #	References
STAT1	GCCTCAAGTTTGCCAGTGGC GGCTCCCTTGATAGAACTGT	*BspHI* 5′T/CATGA3′	314	AW289395	[11]
STAT5A	GAGAAGTTGGCGGAGATTATC CCGTGTGTCCTCATCACCTG	*BstEII* 5′G/GTNACC3′	820	NW_001493678	[15]

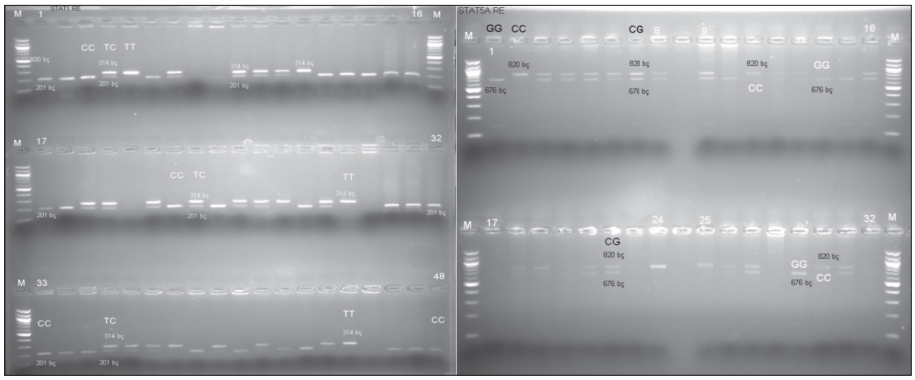


Fig 1. The patterns of restriction fragments of *STAT1* and *STAT5A* genes after digestions with *BspHI* and *BstEII* enzymes, respectively

amplification was carried out by an initial denaturation at 95°C for 5 min, 30 cycles a denaturation at 94°C for 45 s, an annealing at 50°C for 45 s, an elongation at 72°C for 45 s and a final extension at 72°C for 7 min. PCR products were digested with 10 U/µL of each *BspHI* and *BstEII* enzymes (Thermo Fisher Scientific Inc., USA) at 37°C for about four hour to determine allelic polymorphism in *STAT1* and *STAT5A* genes, respectively. A list of the primers used in the amplification of the target gene regions is provided in *Table 1*. A total of 999 cattle, 755 animals from imported dairy breeds and 244 animals from native breeds were genotyped within the scope of this study.

The allelic and genotypic frequencies were calculated with the method of direct gene counting, whether the distributions of genotypic frequencies were following the Hardy-Weinberg genetic equilibrium by the chi-square test. F-statistics are used to compare genetic variability in the total population, intra-subpopulation, and individual structures. These *F* criteria are known as F_{IT}, F_{IS}, and F_{ST}. The F_{IT} value was calculated as the difference of the total actual level of heterozygosity in all populations. The F_{ST} value indicates the genetic diversity among populations. The N_m value was estimated from the F_{ST} value according to the equation $(F_{ST}: 0.25(1-F_{ST})/F_{ST})$ to determine gene flow. The observed values were calculated as the ratio of the genotypes with such a trait to the total number of genotypes, while the expected values were determined, the fixation index (F_{IS}) values, N_e, the effective number of alleles and N_{ei} values were detected, respectively. Furthermore, the unweighted pair group method average *(UPGMA)* analysis was conducted to display the relationship among the bovine breeds phylogenetically [19].

RESULTS

In the study, first of all, the digested PCR products were distinguished by the alleles of *C* and *T* for *STAT1* and the alleles of *C* and *G* for *STAT5A*. The *T* allele was indicated by a band of 314 bp and the *C* allele was indicated by two bands of 201 and 113 bp for *STAT1*. On the other hand; the digestion products were determined by 820 bp for *C* allele and 626 bp for *G* allele in *STAT5A (Fig. 1)*. Based on the results, all breeds were found to be polymorphic for the marker loci in *STAT1* and *STAT5A* genes. The allelic gene and genotypic frequencies, as well as the χ2 results determined for the *STAT1* gene in Jersey, Holstein in BSR, Holstein in MR, Grey Steppe, East Anatolian Red, and Anatolian Black Cattle are presented in *Table 2*. The frequencies of *C* allele of the *STAT1* gene were found as 0.70, 0.74, 0.66, 0.92, 0.86, and 0.82 for Jersey, Holstein in BSR, Holstein in MR, Grey Steppe, East Anatolian Red, and Anatolian Black Cattle breeds, respectively. Of all populations, only Jersey was not in genetic equilibrium due to the various environmental effects, like sampling or inbreeding pressure. In general, the rate of *C* allele was found high for the *STAT1* gene in all populations as compared with *T*.

The allelic gene and genotypic frequencies, as well as the χ2 results determined for the *STAT5A* gene in Jersey, Holstein in BSR, Holstein in MR, Grey Steppe, East Anatolian Red, and Anatolian Black Cattle breeds, are presented in *Table 3*. The frequencies of *C* of the *STAT5A* gene were calculated as 0.79, 0.47, 0.48, 0.39, 0.54, and 0.45 for Jersey, Holstein in BSR, Holstein in MR, Grey Steppe, East Anatolian Red, and Anatolian Black Cattle breeds, respectively. Of all populations, only Grey Steppe breed was not in genetic equilibrium more likely due to the environmental influences over the observed allelic frequencies. In general, the rate of *C* allele was higher than that of *G* allele for the *STAT5A* gene in the populations for Jersey and East Anatolian Red. In case of Holstein, it is almost the same frequency between the alleles but for Grey Steppe and Anatolian Black Cattle breeds the frequencies of *G* allele had higher than that of *C* allele.

Table 2. *The gene, genotypic frequencies, and chi-square results for the STAT1 gene*

Population	Allele #	Allelic Gene Frequency (%)		Genotypic Frequency (%)			χ2
		C	T	CC	CT	TT	
Jersey	566	0.70	0.30	0.43	0.53	0.04	17.25**
Holstein in BSR	326	0.74	0.26	0.55	0.39	0.06	0.08
Holstein in MR	618	0.66	0.34	0.45	0.41	0.14	2.37
Grey Steppe	186	0.92	0.08	0.84	0.16	---	0.66
East Anatolian Red	132	0.86	0.14	0.73	0.26	0.01	0.09
Anatolian Black Cattle	170	0.82	0.18	0.66	0.33	0.01	1.40
Overall	1998	0.73	0.27	0.53	0.40	0.07	0.71

BSR: *Black Sea Region,* ***MR:*** *Marmara Region; ** P<0.01*

Table 3. *The gene, genotypic frequencies, and chi-square results for the STAT5A gene*

Population	Allele #	Allelic Gene Frequency (%)		Genotypic Frequency (%)			χ2
		C	G	CC	CG	GG	
Jersey	496	0.79	0.21	0.63	0.32	0.05	0.56
Holstein in BSR	276	0.47	0.53	0.18	0.59	0.23	4.14*
Holstein in MR	584	0.48	0.52	0.25	0.46	0.29	1.98
Grey Steppe	178	0.39	0.61	0.22	0.34	0.44	7.98**
East Anatolian Red	130	0.54	0.46	0.31	0.46	0.23	0.40
Anatolian Black Cattle	164	0.45	0.55	0.26	0.38	0.36	4.75*
Overall	1828	0.56	0.44	0.34	0.42	0.24	19.93**

BSR: *Black Sea Region,* ***MR:*** *Marmara Region; * P<0.05, ** P<0.01*

Table 4. *The F-statistics results of the STAT1 gene for breeds*

Population	Na[1]	Ne[2]	I[3]	PIC[4]	Obs-Hom[5]	Obs-Het[5]	Exp-Hom[5]	Exp-Het[5]	Ave-Het[6]	Nei[7]	F_{IS}[8]
Jersey	2	1.72	0.61	0.332	0.47	0.53	0.57	0.43	0.53	0.42	-0.248
Holstein in BSR	2	1.61	0.57	0.311	0.60	0.40	0.61	0.39	0.40	0.38	-0.026
Holstein in MR	2	1.81	0.64	0.348	0.58	0.42	0.54	0.46	0.42	0.44	0.086
Grey Steppe	2	1.17	0.28	0.136	0.83	0.17	0.85	0.15	0.17	0.14	-0.087
East Anatolian Red	2	1.32	0.41	0.212	0.74	0.26	0.75	0.25	0.26	0.24	-0.045
Anatolian Black Cattle	2	1.40	0.46	0.252	0.67	0.33	0.71	0.29	0.33	0.29	-0.133
Overall	2	1.63	0.57	0.317	0.60	0.40	0.61	0.39	0.40	0.38	-0.027

[1] Na: Observed number of alleles, [2] Ne: Effective number of alleles,[3] I: Shannon's information index, [4] PIC:P olymorphism Information Content, [5] Observed and expected homozygosity and heterozygosity, repectively, [6] Average heterozygosity, [7] Nei's expected heterozygosity, [8] Fixation index, BSR: Black Sea Region, MR: Marmara Region

The results of the F-statistics determined for the *STAT1* gene in all breeds are presented in *Table 4*. When the F_{IS} values of the populations for the *STAT1* gene were considered, the amount concerned was seen as 8% in Holstein in MR with the dominance of homozygous individuals, while it was displayed as 24% in Jersey, 2% in Holstein in BSR, 8% in Grey Steppe, 4% in East Anatolian Red, and 13% in Anatolian Black Cattle breeds with the dominance of heterozygous individuals. The expected deviations from the Hardy-Weinberg ratio in terms of the *STAT1* locus in these populations were found to be a significant in the Jersey population only (P<0.01). The dominance of heterozygous individuals at a rate of 2% was seen on the general population basis. Expected homozygosity was 57% in Jersey, 61% in Holstein in BSR, 54% in Holstein in MR, 85% in Grey Steppe, 75% in East Anatolian Red, and 71% in Anatolian Black Cattle breeds, with the most homogeneous genes observed in Grey Steppe breed. However, this value was detected as 61% in general. In all populations, the expected heterozygosity was estimated as 42% in Jersey, 38% Holstein in BSR, 44% in Holstein in MR, 14% in Grey Steppe, 24% in East Anatolian Red, and 29% in Anatolian Black Cattle breeds, whereas it was calculated as 38% in general. As a result of the statistical analyses, the highest polymorphism information content *(PIC)* value for *STAT1* gene was observed to be 0.348 for Holstein in MR, and the lowest one was found as 0.136 for Grey Steppe cattle *(Table 4)*. The average *PIC* value for the same gene was 0.317. On the other hand, the highest *PIC* value in terms of *STAT5A* gene was calculated as 0.375 in Holstein in MR, and this value was determined as the lowest of 0.277 in

Table 5. *The F-statistics results of the STAT5A gene for breeds*

Population	Na[1]	Ne[2]	I[3]	PIC[4]	Obs-Hom[5]	Obs-Het[5]	Exp-Hom[5]	Exp-Het[5]	Ave-Het[6]	Nei[7]	F_{IS}[8]
Jersey	2	1.50	0.51	0.277	0.68	0.32	0.67	0.33	0.32	0.33	0.045
Holstein in BSR	2	1.99	0.69	0.374	0.41	0.59	0.50	0.50	0.59	0.49	-0.176
Holstein in MR	2	1.99	0.69	0.375	0.54	0.46	0.50	0.50	0.46	0.49	0.080
Grey Steppe	2	1.91	0.67	0.363	0.66	0.34	0.52	0.48	0.34	0.47	0.293
East Anatolian Red	2	1.98	0.69	0.373	0.54	0.46	0.50	0.50	0.46	0.49	0.071
Anatolian Black Cattle	2	1.97	0.98	0.373	0.62	0.38	0.50	0.50	0.38	0.49	0.234
Overall	2	1.97	0.68	0.371	0.58	0.42	0.51	0.49	0.42	0.49	0.147

[1] Na: Observed number of alleles, [2] Ne: Effective number of alleles, [3] I: Shannon's information index, [4] PIC: Polymorphism Information Content, [5] Observed and expected homozygosity and heterozygosity, repectively, [6] Average heterozygosity, [7] Nei's expected heterozygosity, [8] Fixation index, BSR: Black Sea Region, MR: Marmara Region

Table 6. *The gene flow (N_m) and F-statistics for the STAT1 and STAT5A genes*

Loci	Allele #	F_{IS}	F_{IT}	F_{ST}	N_m*
STAT1	1998	-0.077	-0.019	0.049	4.852
STAT5A	1828	0.092	0.1516	0.0652	3.582

** The N_m value was calculated from the F_{ST} value; F_{ST}: 0.25(1-F_{ST})/F_S*

Jersey breed. The average *PIC* value is 0.371 at this time *(Table 5)*.

The results of the F-statistics determined for the *STAT5A* gene in all breeds are also provided in *Table 5*. When the F_{IS} values of the populations for the *STAT5A* gene were considered, it was seen that the amount concerned was 17% and negative in Holstein in BSR population only; accordingly, the dominance of heterozygous individuals was observed. This value was 4% in Jersey, 8% in Holstein in MR, 29% in Grey Steppe, 7% in East Anatolian Red, and 23% in Anatolian Black Cattle breeds and had a positive sign; furthermore, the dominance of homozygous individuals was displayed. The expected deviations from the Hardy-Weinberg ratio in terms of the *STAT5A* locus in the populations were found significant in Holstein in BSR and Anatolian Black Cattle ($P<0.05$) and Grey Steppe ($P<0.01$) breeds. On general population basis, the dominance of homozygous individuals was found to be at the rate of 14%, and the expected deviations from the Hardy-Weinberg ratio were observed as significant ($P<0.01$). Expected homozygosity was 67% in Jersey, 50% in Holstein in BSR, 50% in Holstein in MR, 52% in Grey Steppe, 50% in East Anatolian Red, and 50% in Anatolian Black Cattle breeds, with the most homogeneous genes shown in Jersey breed. Nevertheless, this value was recorded as 51% in general. In all populations, the expected heterozygosity for the *STAT5A* gene was found as 33% in Jersey, 49% in Holstein in BSR, 49% in Holstein in MR, 47% in Grey Steppe, 49% in East Anatolian Red, 49% in Anatolian Black Cattle breeds and 49% in general. In this study, the average values of heterozygosity for the *STAT1* and *STAT5A* genes were calculated as 0.53, 0.40, 0.42, 0.17, 0.26, and 0.33 as well as 0.32, 0.59, 0.46, 0.34, 0.46, and 0.38 in Jersey, Holstein in BSR, Holstein in MR, Grey Steppe, East Anatolian Red, and Anatolian Black Cattle, respectively.

The results of the gene flow *(N_m)* and F-statistics for the *STAT1* and *STAT5A* genes when both loci analyzed together are given in *Table 6*. According to the F_{ST} value determined for the *STAT1* gene, there was a decrease in the genetic diversity among the subpopulations *(F_{ST}:* 0.049) and this value was 4.9%. According to the F_{IT} value calculated *(F_{IT}:* -0.019), the actual level of heterozygosity in the population for all individuals differed by 1.9% from what it should be according to the Hardy-Weinberg principle. According to the F_{ST} value found for the *STAT5A* gene, the decrease in the genetic diversity among the subpopulations was recorded as F_{ST}: 0.0652. According to the F_{IT} value calculated *(F_{IT}:* 0.1516), the actual level of homozygosity in the population for all individuals differed by about 15% from what it should be according to the Hardy-Weinberg principle.

Genetic similarity and distance values resulting from the analysis of both loci together for the *STAT1* and *STAT5A* genes are presented in *Table 7*. The genetic distance values were found to range from 0.0029 to 0.1599 among the populations. The lowest genetic distance values were detected between East Anatolian Red and Anatolian Black Cattle populations, whereas the highest values were observed between Holstein in BSR and Grey Steppe populations. As a result of the cluster analysis, two clusters occurred for the *STAT1* and *STAT5A* genes *(Fig. 2)*. While Holstein in BSR, Holstein in MR, Anatolian Black Cattle, and East Anatolian Red were found closely clustered together, Grey Steppe was more distant from this main cluster, and Jersey was located in a completely different cluster.

Based on the overall results, *C* allele is more favorable than *T* allele for all breeds in *STAT1* gene. However, *C* allele is more frequent than *G* allele for Jersey and East Anatolian Red, but this is the exact opposite cases for Holstein and other indigenous breeds in *STAT5A*.

DISCUSSION

In the study performed, the frequency of *C* allele was generally found high for the *STAT1* gene in all populations as compared with *T* allele and the ratios of *CC*, *CT*, and *TT* genotypes were found as 53%, 40%, and 0.07%, respectively. In one of the recent studies, the frequencies of *CC*, *CT* and

Table 7. *Genetic similarity and genetic distance for STAT1 and STAT5A genes*

Population	Jersey	Holstein in BSR	Holstein in MR	Grey Steppe	East Anatolian Red	Anatolian Black Cattle
Jersey	-----	0.9561	0.9749	0.9183	0.9950	0.9847
Holstein in BSR	0.0449	----	0.9818	0.8522	0.9797	0.9920
Holstein in MR	0.0254	0.0183	----	0.9360	0.9901	0.9928
Grey Steppe	0.0852	0.1599	0.0662	----	0.9149	0.8986
East Anatolian Red	0.0051	0.0205	0.0099	0.0890	-----	0.9971
Anatolian Black Cattle	0.0154	0.0080	0.0072	0.1069	0.0029	-----

Above the diagonal are the values of genetic similarity, while below it are the values of genetic distance. ***BSR:*** *Black Sea Region,* ***MR:*** *Marmara Region*

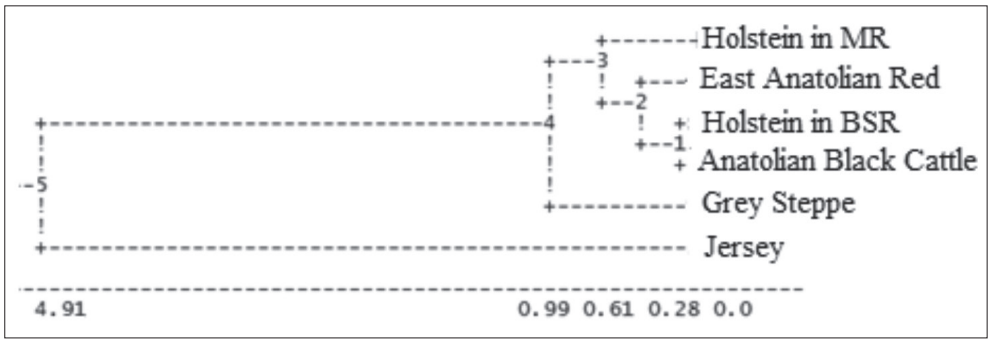


Fig 2. The *UPGMA* dendrogram showing relationships among the populations at the time of two locus process for *STAT1* and *STAT5A* polymorphisms

TT genotypes of the *STAT1* gene were reported similarly in the same pattern but somewhat lower as 45.24% for CC, 36.31% for *CT* genotypes, but a little higher as 18.45% for *TT* genotype in Holstein cows raised in the other farm also located to west part of Turkey, respectively [20]. They reported that the population did not follow Hardy-Weinberg Equilibrium for this locus which might be due to sampling, inbreeding or population stratification.

Many studies on the *STAT5A* gene have been performed in different cattle breeds in various environmental conditions. In these studies several polymorphic sites in bovine *STAT5A* were occupied for an association test with reproductive and productive traits in cow populations [14,16,17]. In a current study, the frequency of *C* allele was generally found high for the *STAT5A* gene in Jersey and East Anatolian Red populations as compared with *G*, which was its alternative allele. In this case, Holstein, Grey Steppe, and Anatolian Black Cattle breeds had higher frequencies of *G* allele than that of *C* allele. For the *STAT5A* gene, the rates of *CG* genotype were generally higher than the other genotypes; on the contrary, *CC* genotype was observed in high frequency in Jersey breed. One of the other studies, the *STAT5A* polymorphism was investigated in Holstein cow [21]. They reported that the population was found in genetic equilibrium. The frequencies of *CC*, *CT*, and *TT* genotypes were reported as 0.751, 0.234 and 0.015 for the gene concerned. Regarding this gene, heterozygosity and effective number of alleles (N_e) were reported as 0.229 and 1.298, respectively. One of the recent study, Oner et al.[22] investigated the effect of *STAT5A* and some other genes on fertility in Holstein-Friesian heifers. Even if the allele frequency of *G* was reported as higher than *C* allele, the association between *STAT5A* polymorphism and fertility was not significantly important. However Ouerghi et al.[23] reported that the substitution of *C* allele by *G* at *STAT5* might be an alternative for improving fertility rate in dairy cows of Tunisia.

The *STAT5A* gene polymorphism was also investigated in the Simmental cattle reared in Romania. For this gene, the frequencies of *CC*, *CT*, and *TT* genotypes were similarly found as 0.67, 0.33, and 0.00, respectively. Moreover, it was expressed that the population was in genetic equilibrium [24]. Arslan et al.[25] investigated the *STAT5A* gene and the other two gene polymorphisms in five different indigenous breeds reared in Turkey. For East Anatolian Red and Anatolian Black Cattle, the frequencies of *CC* genotypes were recorded as 63.1% vs. 62.5% and the frequencies of *C* allele as 71% and 72%; respectively nevertheless both breeds were not found in genetic equilibrium for *STAT5A*. Even if the genotypic frequency of *CC* genotype (75%) and allelic frequency of *C* (86%) was higher in Grey Steppe breed, it was found in genetic equilibrium on the contrary. In a fairly recent study, four different genes including *STAT5A* were searched to identify genetic polymorphism in 167 Turkish Holstein cows. Holstein cows were found to be polymorphic and three genotypes with *CC*, *CT* and *TT* were identified with regard to *SNP-AvaI* polymorphism in the 7^{th} exon of bovine *STAT5A* gene. The *CC* genotype was the most common with 74.2% followed by *CT* with 24% and *TT* with 1.8%, respectively. It was not detected any deviation from Hardy-Weinberg Equilibrium for this locus, either [26].

Based on the different polymorphic site at the position 12.743 in exon 16 for the *STAT5A* gene, the frequencies of *TT*, *CT*, and *CC* genotypes were found to be 0.72, 0.26 and 0.02 in the Polish Friesian herd, respectively [14]. They reported

the frequencies of 0.85 and 0.15 for *T* and *C* alleles in this herd, respectively. On the contrary, the frequencies of *CC*, *TT,* and *CT* genotypes for the *STAT5A* locus were 75.2%, 0.4%, and 24.4% and the *C* and *T* allele frequencies were 0.875 and 0.125 in Chinese Holstein cattle, respectively [27]. The inconsistencies observed among the studies are probably due to differences in cattle breeds. In another study carried out for the *STAT5A* polymorphism at the same chromosomal location in Jersey cattle, the genotypic frequencies for the gene concerned were similar as 73.68%, 23.16% and 3.16% for *TT*, *TC*, and *CC*, respectively and the herd was found in genetic equilibrium [17]. However, when the relationships between the *STAT5A/AvaI* polymorphism at position 6,853 within the exon 7 was investigated in another study; the genotypic frequencies concerning this gene were found as 51.83%, 47.12% and 1.05% for *CC*, *CT*, and *TT* genotypes in Jersey, respectively. The herd was not seen in genetic equilibrium according to the Hardy-Weinberg principle [16].

In contrast to the aforementioned study, allele frequencies in this study were determined as 0.75 for *C* and 0.25 for *T*, respectively. In a recent study, the relationships between the nucleotide polymorphism at position 12.743 in exon 16 of *STAT5A* gene and growth traits were investigated in Podolica bulls by Selvaggi et al.[28]. The frequencies of *TT*, *TC* and *CC* genotypes were recorded as 45.70%, 39.78% and 14.51%, respectively. The observed allele frequencies of *C* and *T* were 0.344 and 0.656, respectively. Unlike a previous study, the herd was found in genetic equilibrium. In another study, Selvaggi et al.[29] investigated two different polymorphic regions at *STAT5A* gene in 92 Agerolese cows belonging 15 different sires in Italy. They reported that all the genotypes of animals were *CC* at *STAT5A/AvaI* locus, there were no any animal with *CT* and *TT* genotypes. On the other hand, the animals with *TT* genotype was the most frequent (75%) followed by the animals with *CT* (25%) and there was no any animal observed with *CC* genotype at *STAT5A/MslI* locus in this herd. The frequencies of *T* and *C* were observed as 0.875 and 0.125, respectively which also indicated that the population was in Hardy-Weinberg Equilibrium for this locus. In a recent study, Coizet et al.[30] investigated the effect of *STAT5A* and two other genes on dairy production in Mediterranean Italian Buffalo. Based on the *SNP* marker polymorphism detected at intron 8-9 in *STAT5A* gene, the buffaloes with *TT* genotypes displayed higher percentages than the buffaloes with other genotypes.

In terms of *STAT1* gene, the observed homozygosity and heterozygosity values were 0.40 and 0.60 in Iranian Holstein cattle. The average value of heterozygosity was found as 0.56 [31]. In the present study, however, the observed homozygosity and heterozygosity values were 0.59 and 0.41, respectively and the average value of heterozygosity was found as 0.41 for *STAT1* in Holstein breed which is quite lower than the study with Iranian Holstein. Since the average value of heterozygosity is unaffected by the sampling error, it is acknowledged as one of the best indicators of genetic diversity [32].

According to the F_{ST} value found for the *STAT1* gene, there was a decrease in the genetic diversity among the subpopulations. Depending on this value, the genetic difference among the subpopulations was also at a low level. According to the F_{IT} value, the actual level of heterozygosity in the population is different than what it should be based on the Hardy-Weinberg. F_{ST} value was merely calculated for the *STAT5A* gene and depending on this value; the genetic difference among the subpopulations was at a moderate level. Based on F_{IT} value, the actual level of homozygosity in the population was different than what it should be according to the Hardy-Weinberg by 15%. In one of the nearly conducted study; various gene polymorphisms including *STAT5A* were investigated in Turkish native cattle breeds [25]. The F_{ST} values between the breeds for *STAT5A* were reported as 0.006 between Anatolian Black Cattle and East Anatolian Red, as 0.014 between Anatolian Black Cattle and Grey Steppe, and as 0.009 between East Anatolian Red and Grey Steppe which the results were not concordant to the findings of this current study.

When both loci were considered together, the lowest genetic distance values for the *STAT1* and *STAT5A* genes were between East Anatolian Red and Anatolian Black Cattle populations, whereas the highest values were between Holstein in BSR and Grey Steppe populations. These breeds were aggregated in two main clusters for the *STAT1* and *STAT5A* polymorphisms. Whilst Holstein in BSR, Holstein in MR, Anatolian Black Cattle, and East Anatolian Red were closely clustered together, the Grey breed was clustered separately from the main cluster. Jersey was most distant from all other breeds. Ozbeyaz et al.[33] identified three main clusters in their cluster analysis; South Anatolian Red and Jersey breeds formed two distant clusters and a third cluster between these two clusters was comprised of Brown Swiss, Holstein, East Anatolian Red, Anatolian Black Cattle, and Grey Steppe populations, similar to the findings in the present study.

With this study, the intention was to reveal the genetic diversity in terms of the *STAT1* and *STAT5A* genes and the genetic relationships among the breeds in the populations of two different dairy breeds; Holstein from Black Sea and Marmara Regions and Jersey, as well as of Anatolian Black Cattle, East Anatolian Red, and Grey Steppe out of other native cattle breeds. According to the results obtained about genetic variation among the breeds in terms of *STAT* gene families in this study, it is possible to make genetic progress for these breeds raised in Turkey based on animal selection methods like the Marker Assistant Selection (MAS). But it seems reasonable to continue study applying haplotype analysis by using various polymorphic regions, especially in *STAT5A* gene before using them in Turkish dairy selection programs extensively.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgment

This study was conducted within the scope of the ethical committee decision number: 2010/6-05 dated 29.07.2010, by the Local Ethical Committee for Animal Experiments at Namik Kemal University. The study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with project number #110O821.

REFERENCES

1. Anonymous: Domestic animal genetic resources in Turkey. Ministry of Food Agriculture and Livestock General Directorate of Agricultural Research and Policy. December Issues. Ankara, Turkey, 2011. https://www.animalgeneticresources.net/wp-content/uploads/2018/06/TU_AnGR.pdf; *Accessed:* 03.01.2019.

2. Grechko VV: Molecular DNA markers in phylogeny and systematics. *Russ J Genet,* 38 (8): 851-868, 2002.

3. Darnell JE: Stats and gene regulation. *Science,* 277 (5332): 1630-1635, 1997. DOI: 10.1126/science.277.5332.1630

4. Band MR, Larson JH, Rebeiz M, Green CA, Heyen DW, Donovan J, Windish R, Steining C, Mahyuddin P, Womack JE, Lewin HA: An ordered comparative map of the cattle and human genomes. *Genome Res,* 10 (9): 1359-1368, 2000. DOI: 10.1101/gr.145900

5. Seyfert HM, Pitra C, Meyer L, Brunner RM, Wheeler TT, Molenaar A, McCracken JY, Herrmann J, Thiesen HJ, Schwerin M: Molecular characterization of STAT5A and STAT5B encoding genes reveals extended intragenic sequence homogeneity in cattle and mouse and different degrees of divergent evolution of various domains. *J Mol Evol,* 50 (6): 550-561, 2000. DOI: 10.1007/s002390010058

6. Khatkar MS, Thomson PC, Tammen I, Raadsma HW: Quantitative trait loci mapping in dairy cattle. Review and meta-analysis. *Genet Sel Evol*, 36 (2): 163-190, 2004. DOI: 10.1186/1297-9686-36-2-163

7. Kwon JM, Goate AM: The candidate gene approach. *Alcohol Res Health,* 24 (3): 164-168, 2000.

8. Watson CJ: Stat transcription factors in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia*, 6 (1): 115-127, 2001. DOI: 10.1023/A:1009524817155

9. Boutinaud M, Jammes H: Growth hormone increases STAT5 and STAT1 expression in lactating goat mammary gland: A specific effect compared to milking frequency. *Domest Anim Endocrinol*, 27 (4): 363-378, 2004. DOI: 10.1016/j.domaniend.2004.04.002

10. Rychtarova J, Sztankoova Z, Kyselova J, Zink V, Stipkova M, Vacek M, Stolc L: Effect of *DGAT1, BTN1A1, OLR1* and *STAT1* genes on milk production and reproduction traits in the Czech Fleckvieh breed. *Czech J Anim Sci*, 59 (2): 45-53, 2014. DOI: 10.17221/7228-CJAS

11. Cobanoglu O, Zaitoun I, Chang YM, Shook GE, Khatib H: Effects of the Signal Transducer and Activator of Transcription 1 *(STAT1)* gene on milk production traits in Holstein dairy cattle. *J Dairy Sci*, 89 (11): 4433-4437, 2006. DOI: 10.3168/jds.S0022-0302(06)72491-2

12. Oprazadek J, Flisikowski K, Zwierzchowski L, Dymnicki E: Polymorphisms at *loci* of leptin *(LEP), Pit1* and *STAT5A* and their association with growth, feed conversion and carcass quality in Black and White bulls. *Anim Sci Pap Rep,* 21 (3): 135-145, 2003.

13. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA: Prolactin (PRL) and it's receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev,* 19 (3): 225-268, 1998. DOI: 10.1210/edrv.19.3.0334

14. Flisikowski K, Strzalkowska N, Sloniewski K, Krzyzewki J, Zwierzchowsk L: Association of a sequence nucleotide polymorphism in exon 16 of the *STAT5A* gene with milk production traits in Polish Black-and-White (Polish Friesian) cows. *Anim Sci Pap Rep,* 22 (4): 515-522, 2004.

15. Khatib H, Monson RL, Schutzkus V, Kohl DM, Rosa GJM, Rutledge JJ: Mutation in the *STAT5A* gene are associated with embryonic survival and milk composition in cattle. *J Dairy Sci*, 91 (2): 784-793, 2008. DOI: 10.3168/jds.2007-0669

16. Dario C, Selvaggi M: Study on the *STAT5A*/AvaI polymorphism in Jersey cows and association with milk production traits. *Mol Biol Rep,* 38 (8): 5387-5392, 2011. DOI: 10.1007/s11033-011-0691-8

17. Selvaggi M, Tufarelli V, Pinto F, Centoducati G, Dambrosio A, Santacroce MP, Dario C: Bovine *STAT5A* gene polymorphism analysis and its association with milk composition traits in Jersey cows. *Int J Biosci Biochem Bioinform,* 3 (4): 341-344, 2013.

18. Sambrook J, Russel DW: Molecular cloning: A laboratory manual. 3rd ed., 999, Cold Spring Harbor Laboratory Press, New York, 2001.

19. Yeh F, Yang RC, Boyle T: PopGene (v.1.32), Microsoft Windows-based freeware for population genetic analysis. Molecular Biology and Biotechnology Centre. University of Alberta, Edmonton, 2000. https://sites.ualberta.ca/~fyeh/popgene.html; *Accessed*: 15.01.2019.

20. Ardicli S, Soyudal B, Samli H, Dincel D, Balci F: Effect of *STAT1, OLR1, CSN1S1, CSN1S2,* and *DGAT1* genes on milk yield and composition traits of Holstein breed. *R Bras Zootec,* 47:e20170247, 2018. DOI: 10.1590/rbz4720170247

21. He X, Chu MX, Qiao L, He JN, Wang PQ, Feng T, Di R, Cao GL, Fang L, An YF: Polymorphisms of *STAT5A* gene and their association with milk production traits in Holstein cows. *Mol Biol Rep,* 39 (3): 2901-2907, 2012. DOI: 10.1007/s11033-011-1051-4

22. Öner Y, Yılmaz O, Okut H, Ata N, Yılmazbaş-Mecitoğlu G, Keskin A: Associations between *GH, PRL, STAT5A, OPN, PIT-1, LEP* and *FGF2* polymorphisms and fertility in Holstein-Friesian heifers. *Kafkas Univ Vet Fak Derg*, 23 (4): 527-534, 2017. DOI: 10.9775/kvfd.2016.17192

23. Ouerghi S, Jemmalı B, Trab I, Gara A, Rekik B: Identification of polymorphism at the *STAT5* locus in dairy cows in Tunisia. *J New Sci – Sust Liv Manag*, 1 (3): 11-14, 2017.

24. Cosier V, Croitoriu V: Research concerning the polymorphic expression of Pit-1 and STAT5A genes in cattle. *Bulletin UASVM Anim Sci Biotechnol*, 69 (1-2): 70-79, 2012. DOI: 10.15835/buasvmcn-asb:69:1-2:8391

25. Arslan K, Akyuz B, Korkmaz Agaoglu O: Investigation of *STAT5A, FSHR,* and *LHR* gene polymorphisms in Turkish indigenous cattle breeds (East Anatolian Red, South Anatolian Red, Turkish Grey, Anatolian Black, and Zavot). *Russ J Genet*, 51 (11): 1088-1095, 2015. DOI: 10.1134/s1022795415110022

26. Kiyici JM, Arslan K, Akyuz B, Kaliber M, Aksel EG, Cinar MU: Relationships between polymorphisms of growth hormone, leptin and myogenic factor 5 genes with some milk yield traits in Holstein dairy cows. *Int J Dairy Technol*, 72 (1): 1-7, 2019. DOI: 10.1111/1471-0307.12539

27. Bao B, Zhang C, Fang X, Zhang R, Gu C, Lei C, Chen H: Association between polymorphism in *STAT5A* gene and milk production traits in Chinese Holstein cattle. *Anim Sci Pap Rep*, 28 (1): 5-11, 2010.

28. Selvaggi M, Gabriellai A, D'Alessandro AG, Dario C: Bovine *STAT5A* gene polymorphism and its influence on growth traits in Podolica breed. *Anim Prod Sci*, 56 (7): 1056-1060, 2016. DOI: 10.1071/AN14739

29. Selvaggi M, Albarella S, Dario C, Peretti V, Ciotola F: Association of *STAT5A* gene variants with milk production traits in Agerolese cattle. *Biochem Genet,* 55 (2): 158-167, 2017. DOI: 10.1007/s10528-016-9781-6

30. Coizet B, Frattini S, Nicoloso L, Iannuzzi L, Coletta A, Talenti A, Minozzi G, Pagnacco G, Crepaldi P: Polymorphism of the *STAT5A, MTNR1A* and *TNFa* genes and their effect on dairy production in *Bubalus bubalis*. *Ital J Anim Sci*, 17 (1): 31-37, 2018. DOI: 10.1080/1828051X.2017.1335181

31. Askari GH, Ansari Mahyari S, Rahimi Mianji G, Nanaei HA: Effect of *STAT1* variants on milk production traits in Esfahan Holstein Cows. *Int J Adv Biol Biomed Res,* 1 (8): 851-857, 2013.

32. Savaşçı M, Atasoy F: The investigation of calpastatin and thyroglobulin gene polymorphisms in some native cattle breeds. *Ankara Üniv Vet Fak Derg*, 63 (1): 53-59, 2016. DOI: 10.1501/Vetfak_0000002709

33. Özbeyaz, C, Yıldız, MA, Çamdeviren H: Türkiye'de yetiştirilen çeşitli sığır ırkları arasındaki genetik ilişkiler. *Lalahan Hay Araşt Enst Derg*, 39 (1): 17-32, 1999.

Kafkas Univ Vet Fak Derg
26 (2): 263-268, 2020
DOI: 10.9775/kvfd.2019.22925

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Dose- and Time-dependent Effects of Permethrin on HepG2 Cells: Cell Survival, Lipid Peroxidation and Antioxidant Defence System [1] [2]

Dilek GUVENC [1,a] M. Yavuz GULBAHAR [2,b] Oguzhan YAVUZ [1,c] Tolga GUVENC [2,d]

[1] *This project was supported by Ondokuz Mayis University Research Foundation (Project No PYO.VET.1901.15.008)*

[2] *The results of this study were partially presented as an abstract at the 17th International Symposium" Prospects for the 3rd Millennium Agriculture", Cluj-Napoca, Romania, September 27 to 29, 2018 and "International Congress on Engineering and Life Sciences-ICELIS" Kastamonu, Turkey, 26 to 29 April 2018*

[1] Department of Pharmacology and Toxicology. Faculty of Veterinary Medicine, University of Ondokuz Mayis, TR-55139 Samsun - TURKEY

[2] Department Pathology. Faculty of Veterinary Medicine, University of Ondokuz Mayis, TR-55139 Samsun - TURKEY

[a] ORCID: 0000-0003-0036-0914: [b] ORCID: 0000-0001-8268-7659: [c] ORCID: 0000-0002-1419-1464; [d] ORCID: 0000-0003-1468-3415

Article ID: KVFD-2019-22925 Received: 30.06.2019 Accepted: 25.11.2019 Published Online: 25.11.2019

How to Cite This Article

Guvenc D, Gulbahar MY, Yavuz O, Guvenc T: Dose- and time-dependent effects of permethrin on HepG2 cells: Cell survival, lipid peroxidation and antioxidant defence system. *Kafkas Univ Vet Fak Derg,* 26 (2): 263-268, 2020. DOI: 10.9775/kvfd.2019.22925

Abstract

There is very limited knowledge about *in vitro* hepatotoxicity of permethrin concerning dose and duration even though humans and non-targeted beings are exposed. In this study, three different doses of permethrin (1 uM, 10 uM, 100 uM) were administered in three different time periods (24, 48, 72 h) and cell viability (WST-1 and Trypan blue test), lipid peroxidation (high performance lipid chromatography), and antioxidant (SOD-1, SOD-2 and GPx-1) gene expression levels (real time PCR) were evaluated. The LC50 dose of permethrin was calculated as 1111 µM. Significant decrease in cell viability was detected in every time period except at the lowest dose ($P<0.05$). Each permethrin dose caused a significant increase ($P<0.01$) in superoxide dismutase-1 levels (except 1 µM at 48 h). The 10 µM and 100 µM groups' superoxide dismutase-2 levels were higher than the controls at each exposure level though the 1 µM group was significantly lower at 24 and 48 h and higher at 72 h. Interestingly, a non-uniform statistically significant difference for glutathione peroxidase-1 was seen in each exposure duration and doses either as up- or down regulation ($P<0.01$). Generally, malondialdehyde concentrations were significantly increased ($P<0.01$), although at each dose in 72 h a significant decrease in malonaldehyde levels was seen ($P<0.01$). Our results may help in understanding the molecular aspects of high dose permethrin hepatotoxicity. More comprehensive research is required to evaluate long term low dose exposure.

Keywords: *Cytotoxicity, HepG2, Oxidative stress, Permethrin*

Permetrin'in HepG2 Hücrelerine Doza ve Zamana Bağlı Etkileri: Hücre Sağkalımı, Lipid Peroksidasyonu ve Antioksidan Savunma Sistemi

Öz

Permetrinin tüm dünyada yoğun kullanımıyla insanlar ve diğer hedef dışı canlılar maruz kalmasına ragmen doz ve zamana bağlı *in vitro* hepatotoksisitesi ile ilgili sınırlı bilgi bulunmaktadır. Bu çalışmada, HepG2 hücrelerine permetrinin üç farklı dozu (1 uM, 10 uM, 100 uM) üç farklı zaman aralığında (24, 48, 72 saat) uygulanarak hücre canlılığı (WST-1 ve Tripan Mavisi testi ile), lipid peroksidasyon (yüksek performanslı sıvı kromatografisi ile), ve antioksidan (SOD-1, SOD-2 ve GPx-1) gen ekspresyon düzeylerinin (gerçek zamanlı PCR ile) değerlendirilmesi amaçlanmıştır. Permetrinin LC50 dozu 1111 µM olarak hesaplandı. Tüm zamanlarda en düşük doz hariç hücre canlılığında önemli azalma belirlendi ($P<0.05$). Permetrin'in tüm dozları, tüm maruz kalma sürelerinde (48 saatte 1 µM hariç) süperoksit dismutaz-1 seviyelerinde önemli bir artışa neden oldu ($P<0.01$). Tüm maruziyet sürelerinde 10 µM ve 100 µM grublarında süperoksit dismutaz-2 seviyeleri kontrol grubundan daha yüksek olmasına rağmen, 1 µM grubu 24 saat ve 48 saatte anlamlı olarak düşüktü ve 72 saatte daha yüksekti ($P<0.01$). İlginç olarak Glutatyon peroksidaz-1 için, tüm maruziyet grupları ve maruz kalma sürelerinde kontrole göre uniform olmayan up ya da down regülasyon şeklinde istatistiksel olarak anlamlı fark gözlendi ($P<0.01$). Genel olarak, malondialdehit konsantrasyonları önemli ölçüde artmasına karşın ($P<0.01$), tüm permetrin dozlarında 72 saatte malondialdehit seviyelerinde önemli bir düşüş olmuştur ($P<0.01$). Sonuçlarımız yüksek doz permetrin kaynaklı hepatotoksisitede rol oynayan moleküler yönlerin daha iyi anlaşılmasını sağlayabilir. Düşük doz uzun süreli maruziyet için daha detaylı araştırmalara ihtiyaç vardır.

Anahtar sözcükler: *Sitotoksisite, HepG2, Oksidatif stres, Permetrin*

İletişim (Correspondence)
+90 362 3121919
tguvenc@omu.edu.tr

INTRODUCTION

Permethrin (PER), a synthetic pyrethroid insecticide is commonly used for many purposes, such as agriculture, public health, forestry, veterinary medicine, and vector control programs, due to its high activity as an insecticide and low mammalian toxicity [1,2]. Approximately 2 million pounds of PER is used annually in the fields of agriculture, housing and public health, mostly in non-agricultural areas (70%) [3]. However, this extensive use increases its residual contamination in the environment and potential for human and animal exposure [2]. In addition, a number of studies have confirmed its carcinogenic potential [4,5] as well as generation of oxidative damage [2] in vertebrates and invertebrates.

Permethrin acts on the nervous system of target organisms. It forms damage in function of voltage-gated sodium channels in the neurons, and causes paralyses and eventually death with muscular spasm. Compared to those of insects, mammalian sodium channels are less sensitive to effects of pyrethroids because of rapid recovering [6]. Also, pyrethroids have low hydrolytic activity, and they are easily converted to non-toxic derivatives thorough hydrolytic activity in mammalian species in contrast to insects [7].

The liver is an important organ in the detoxification of xenobiotics, and it is known that PER is a potent inhibitor for cytochrome P4501A, resulting in substantial accumulation of some chemicals associated with fatal toxicities [8,9]. PER also causes some pathological alterations in hepatocytes, reducing the size of the nuclei and causing hydropic degeneration of the hepatocytes [10]. A previous study showed imbalance and damage in the redox system of the liver of rats in adolescence (subchronic) and neonatal (subacute) age following PER treatment [8].

Although the action of pyrethroids on various target cells is different, oxidative stress (OS), reactive oxygen species (ROS) and reactive nitrogen species (RNS) associated with PER may play critical roles in induction of a variety of toxicities in humans and animals, including neurotoxicity [11], immunotoxicity [12], cardiotoxicity [13], hepatotoxicity [8,10,14,15], genotoxicity [16], and cytotoxicity [10,17]. It has been shown that PER induces a significant increase in OS in various types of cells such as erythrocytes, leukocytes, brain cells, heart cells, smooth muscle cells, thymic cells and lymphocytes [2].

HepG2 is a human hepatocellular carcinoma cell line with high proliferation capacity and many differentiated hepatic functions that can metabolically transform initial substances by their endogenously overexpressed oxygenases [18]. HepG2 cell monoculture or co-culture models have been most commonly used in drug metabolism and hepatotoxicity studies for the prediction of pesticide-induced liver injury, as *in vitro* alternative to primary human hepatocytes [18-21].

A literature search revealed there is no report regarding OS associated with PER in the HepG2 cell line. In the present study, investigation of PER induced OS in human hepatoma HepG2 cells for further understanding the mechanisms of PER toxicity was aimed. The effect of different doses of PER on cell viability, lipid peroxidation and antioxidant enzymes were assessed in a time-dependent manner. For this purpose, the measurement of malondialdehyde (MDA) level as a marker of lipid peroxidation was analysed by high performance lipid chromatography (HPLC); and the gene expression of superoxide dismutase-1 (SOD-1), superoxide dismutase-2 (SOD-2) and glutathione peroxidase-1 (GPX-1) were detected by real time PCR.

MATERIAL and METHODS

Cell Culture, WST Assay and Treatment

The human HepG2 hepatocellular carcinoma cell line was provided by American Type Culture Collection (ATCC, HB-8065, Manassas, VA, USA). The cells were grown in Eagle's Minimum Essential Medium (EMEM) (M0275, Sigma, USA) which was supplemented with 10% fetal bovine serum (FBS) (10500064, Gibco), 2 mM L-glutamine (G7513, Sigma USA), 100U/mL penicillin/streptomycin (15140122, Gibco) and 1% non-essential amino acids (M7145, Sigma USA) at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

Firstly, PER (25:75 is a mixture of cis and trans isomers, 3-phenoxybenzyl (IRS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl) -2,2-dimethylcyclopropanecarboxylate, 100% purity, Y0001733, European Pharmacopoeia, Strasbourg, France) was dissolved in dimethyl sulfoxide (DMSO, D2650, Sigma Chemical Co., St. Louis, MO, UK) at a series stock solutions concentration of 1, 10 and 100 mM. The stock solutions were diluted using culture medium in the range of 1-10000 µM. Lethal concentration50 (LC50) of PER was calculated using a colorimetric assay for 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1, 05015944001, Roche Diagnostics, Germany) reagent. For WST-1 assays, HepG2 cells were subcultured in 96-well plates at a seeding density $3x10^4$ cells per well for 24 h. After incubation the medium was changed and the cells were treated with 100 µL different concentrations of PER for 24 h. Additionally each plate contained blanks, controls (no treatment), and vehicle (DMSO, 0.1%) with three replicates each. Then, 10 µL of WST-1 reactant was added to each well and incubated for 30 min. Cell viability was measured at 450 nm in a microplate reader spectrophotometer (Tecan software Magellan, Switzerland).

Cells ($3x10^5$, total cell count of per mL) were treated with three different doses of PER 1 µM, 10 µM and 100 µM (approximately 1/1000, 1/100 and 1/10 of the determined LC50) and at three different time intervals (24, 48 and 72 h). The treatments were performed as three replicates and all the experiments were repeated four times.

HepG2 Cell Viability Assay

The trypan blue exclusion test was used to determine the number of viable cells. Briefly, HepG2 cells were treated with three different doses of PER (1 µM, 10 µM and 100 µM) and at three different time intervals (24, 48 and 72 h) as well as control (no treatment) and DMSO (0.1% vehicle) treatment. Cells were harvested, and then aliquot of 10 µL of the cell's suspension was added with similar volume of 0.4% trypan blue solution (T8154, Sigma-Aldrich, St Louis, MO, USA). Cell viability was analyzed using an automated cell counter (TC-20™, Bio-Rad Laboratories, Hercules, CA, USA).

Lipid Peroxidation Measurements

MDA levels in the cell supernatant were determined with 2,4-dinitrophenylhydrazine (DNPH, D199303) derivative method by HPLC [22]. All analytical grade chemicals were provided by Sigma-Aldrich, St Louis, MO, USA. External standards were prepared in the range of 0.3-10 nmol/mL using 1,1,3,3-tetraethoxypropane (TEP, T9889) in 1% sulphuric acid. Cell supernatant samples (125 µL) were treated with 25 µL 6M aqueous sodium hydroxide and the mixture was incubated at 60°C for 30 min. For precipitation of proteins, 35% perchloric acid was added and centrifuged at 2800 g for 10 min. The supernatant was transferred to the eppendorf tube and then added the DNPH prepared in hydrochloric acid. This mixture was allowed to incubate for 30 min at room temperature in a dark environment. An aliquot of 50 µL of this reaction mixture was injected into the HPLC equipment (Shimadzu, LC-20AT Prominence, Kyoto, Japan) with a photodiode array detector (SPD-M20A, 310 nm). The separation was performed with an ACE 5 C18 column (4.0×125 mm x 5 µm particle size (ACT, Scotland). The mobile phase consisted of 0.2% acetic acid in deionised water (v/v) and acetonitrile/water (62:38, v/v). The flow rate was 0.6 mL/min. The linearity was satisfactory (R^2 = 0.999) and the limit of detection (LOD) and limit of quantification (LOQ) values were 0.003 nmol/mL and 0.01 nmol/mL, respectively. Determination of the protein content was based on the Bradford method [23], using the Bradford reagent (B6916). MDA level was expressed per mg protein.

mRNA Quantification of Antioxidant Enzymes (SOD-1, SOD-2 and GPX-1)

Total RNA was isolated using the RNA Isolation Kit (PureLink RNA mini kit, 12183018A, Ambion), according to the manufacturer's instructions. Purity and concentration of the isolated RNA were detected by measuring absorption at 260-280 nm spectrophotometrically. cDNA synthesis was performed with High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems), according to the manufacturer's instructions. cDNA samples were stored at -20°C until use. Quantitative RT-PCR was performed in triplicate with the LightCycler 1.5 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler TagMan Master Mix and Real Time ready single assays primer-probe sets (04735536001, Roche Diagnostics, Mannheim, Germany). The RT-PCR conditions consisted of initial denaturation phase at 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for one sec. Analysis and quantification were performed using LightCycler 480 software. Relative quantification was calculated by delta Ct method, and SOD-1, SOD-2 and GPX-1 expressions were normalized with respect to the expression of an endogenous control, beta-actin.

Statistical Analysis

The normality of the data was evaluated with the Kolmogorov-Smirnov Test. For all parameters, comparisons of the doses in each experiment time were made with One-Way Analysis of Variance (ANOVA) followed by Tukey's and Duncan tests. All data were expressed as mean ± standard error (SEM). Differences were considered statistically significant when P values were less than 0.05.

RESULTS

WST-1 Assay

Between the vehicle-treated (DMSO) and control cells, there were no significant differences for cell viability. PER resulted in a significant decrease in cell viability in a concentration-dependent manner compared to the control group *(Fig. 1)*. The LC50 value was calculated as 1111 µM for PER.

Cell Viability

When evaluating the results of the trypan blue test for cell viability, there was a statistically significant reduction in cell viabilities at 10 µM and 100 µM PER doses for all three-exposure times (P<0.05). However, the 1 µM treatment dose did not induce significant cell loss compared to

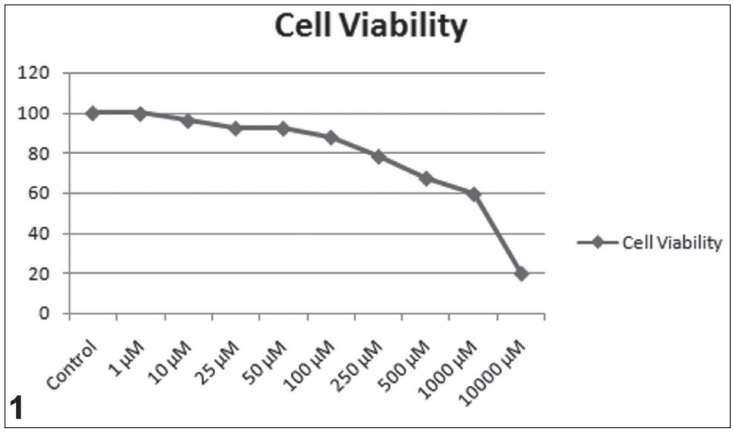


Fig 1. Cytotoxicity of HepG2 cell culture after 24 h exposure time to different concentration of permethrin. Each point represents the average of three independent experiments on cultures of HepG2 cells. Means are plotted

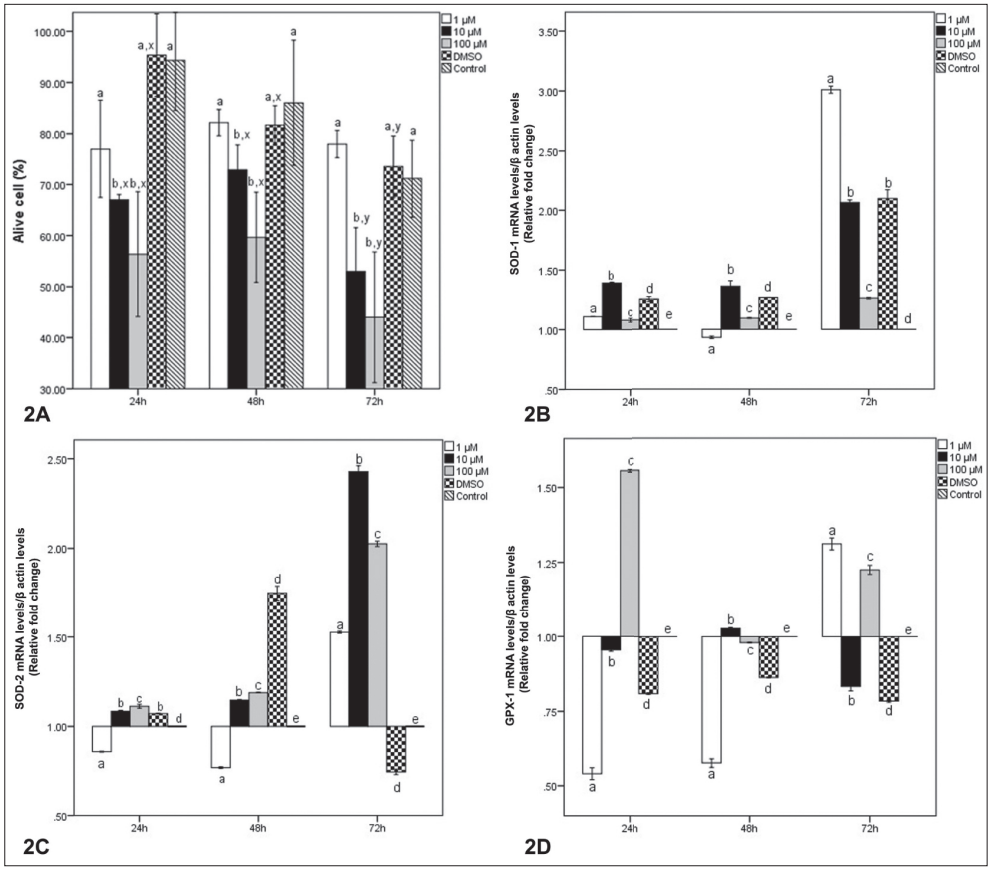


Fig 2. Effects of Permethrin on cell viability and the level of each amplified cDNA was normalized to that of B-actin and represented as the bar graph. A) Cell viability ([a,b] Statistical comparisons of alive cell ratio of groups in each experimental time, [x,y] Time-dependent changes of each group on alive cell ratio, P<0.01) B) SOD-1 mRNA levels C) SOD-2 mRNA levels D) GPX-1 mRNA levels ([a,b,c,d,e] Statistical comparisons of fold changes of mRNA of groups in each experimental time, P<0.01)

Table 1. *MDA (malondialdehyde) levels in PER (permethrin) treated HEPG2 cell line*

Dose	MDA Level (nmol/mg protein)		
	24 h	48 h	72 h
1 µM	0.266±0.015[a]	8.566±0.025[a]	1.253±0.045[a]
10 µM	0.223±0.015[b]	6.466±0.135[b]	0.800±0.040[b]
100 µM	1.853±0.025[c]	5.503±0.075[c]	0.380±0.060[c]
DMSO	0.050±0.01[d]	1.113±0.123[d]	1.413±0.075[d]
Control	0.020±0.01[d]	0.490±0.081[e]	1.516±0.080[e]

Data are expressed as mean ± SEM; [a,b,c,d,e] Means with different superscript in the same column differ significant (P<0.01)

the control and DMSO groups (P>0.05). Time-dependent changes on the cell viability were significantly different in 10 µM, 100 µM and DMSO groups at 72 h (P<0.05) *(Fig. 2-A)*.

Lipid Peroxidation

A significant increase in the MDA concentration of the PER groups was observed at 24 h and 48 h (P<0.01). However, there was a significant decrease in MDA levels at 72 h in all PER doses compared to control and DMSO groups (P<0.01). The alterations in the MDA concentrations were not dose-dependent and the highest level of MDA was detected at the 1 µM dose for all exposure times *(Table 1)*.

mRNA Expression of Antioxidant Enzymes (SOD-1, SOD-2 and GPX-1)

In general, statistically important differences were observed between OS parameters of all treatment groups at all exposure times (P<0.01). However, there was no difference between 10 µM and DMSO groups at certain times (24 h for SOD-2 and 72 h for SOD-1) (P>0.05).

All doses of PER caused a significant increase (up regulation) in SOD-1 levels at all exposure times except 1 µM (down-regulation) at 48 h compared to controls (P<0.01) *(Fig. 2-B)*. SOD-2 levels of the 10 µM and 100 µM groups were upregulated at all exposure times. However, SOD-2 levels of the 1 µM group were significantly downregulated at 24 h and 48 h and upregulated at 72 h compared to those of control groups (P<0.01) *(Fig. 2-C)*. Although, compare to control groups for expression of GPX-1 statistically significant difference (up-or down regulated) were observed in all treatment groups (P<0.01), these expression levels did not have uniform distribution *(Fig. 2-D)*.

In terms of changes in SOD-1, SOD-2 and GPX-1 levels of groups at three exposure times, time-dependent changes (up or down regulation) were observed for all PER groups and the DMSO group. These changes were determined statistically important in all doses (except 1 µM) at all exposure times for SOD-2 and GPX-1 and only at 72 h for SOD1 ($P<0.01$).

DISCUSSION

Permethrin, one of the type I synthetic pyrethroid insecticides, has been widely used in the world due to its high insecticide activities and low toxicity for mammals. However, increasing studies indicate that PER-related toxic effects arise from OS in various toxicity models under many *in vitro* and *in vivo* conditions [2]. In addition, several studies reported that PER and other synthetic pyrethroids have cytotoxic effects on different cell lines in certain doses [10,21,24]. Similarly, PER has dose-dependent cytotoxic effects on HepG2 cells in the present study. The alive cell ratio of 1 µM dose was not different from the DMSO and control groups and any cytotoxicity were not determined at the lowest dose. However, the 10 µM and 100 µM doses caused significant cytotoxicity and this effect was maximally observed at 72 h time period.

Lipid peroxidation, damage to cell membranes, lipoproteins, and other lipid-containing structures triggered by OS and generation of ROS or RNS, is a well-known process associated with various pathological conditions and accelerated aging, leading to molecular cell damage, such as necrosis or apoptosis [25].

Cells in response to membrane lipid peroxidation either stimulate survival or induce cell death processes, and this situation depends on cellular metabolic conditions and repair capacities. In the case of low lipid peroxidation (sub-toxic conditions), cells stimulate survival by providing adaptive stress responses, such as antioxidant defence systems or signalling pathways. In the case of moderate or high lipid peroxidation (toxic conditions), oxidative damage exceeds repair capacity and cell death, such as apoptosis/autophagy/necrosis, is induced. In the present study, adaptive responses were insufficient in 10 µM and 100 µM doses at 72 h, these situations could be explained by low number of alive cells. The peroxidation of lipids usually takes place in three stages: initiation, propagation and termination. In the early stages, lipid radicals and lipid hydroperoxides occur, whereas in later stages, aldehyde products such as MDA are formed [26,27]. In our study, an increase in MDA levels was observed in all doses from 24 h to 48 h, which may indicate that the 24 h and 48 h are the early stage and the late stage of lipid peroxidation, respectively.

Lipid peroxidation yields a wide variety of oxidation products including many different aldehydes, such as MDA, propanal, hexanal, and 4-hydroxynonenal. MDA is one of the final products of polyunsaturated fatty acids, such as omega-3 and omega-6 fatty acids, and is accepted as a convenient lipid peroxidation marker [25,28]. In a rat model of dermal PER exposure, significant increases in plasma MDA concentrations are associated directly with tissue damage [29]. In general, MDA levels of PER groups were determined to be higher than the DMSO and control groups in this study. Increased MDA was not dose-dependent and the highest MDA levels were observed in 100 µM and 1 µM doses at 24 h and 48 h, respectively. In contrast, MDA levels of PER groups were significantly lower than the DMSO and control at groups 72 h. Previous studies showed that PER and analogues (its enantiomers) caused dose-dependent increases of MDA in cell lines [17,20,30]. In our study, increased MDA could be assessed as the lipid peroxidation effect of PER, as well. However, the impact of doses on the MDA level was not observed at 24 h and 48 h. Possible explanations for decreases in the MDA levels at 72 h post-exposure is that it was enzymatically metabolized or it might react on cellular proteins or DNA to form adducts, resulting in damage at the molecular level 72 h after PER exposure [25]. In our study, cell viability results showed that cytotoxicity (via apoptosis and/or autophagy) was observed predominant in time and decreased alive cell ratios could be linked to decreased production of MDA. Also, MDA may bind with some other cellular products, and therefore, it can not be visible after a certain time. In addition, extra stress caused by several changes of the cell medium may cause the increased MDA levels of the DMSO and control groups at 72 h [25-27,30].

Oxidative stress is the imbalance between harmful ROS and the antioxidant defence system. ROS can damage DNA, protein and membrane lipids and cause aging, cancer and various diseases. There is a close link between cellular responses to OS and antioxidant enzyme levels. Commonly used enzymes for evaluation of the OS status are SOD and GPX. Effects of synthetic pyrethroids on OS parameters have been investigated in several papers, and it was reported that single or combined exposure to synthetic pyrethroids resulted in *in vitro* OS [17,20,27,31]. Similar with MDA levels, a general increase in antioxidant enzyme levels was observed in the present study. Also, dependence on the dose was not determined at each exposure time. The obtained findings may be evaluated, as PER caused OS in HepG2 cells, but detailed studies should be performed for investigation of the effects of different doses and time periods. *In vivo* studies also showed that PER dose-dependently induces OS in rat liver, reducing antioxidant enzyme activities including SOD and GPX, and finally leading to the lipid and protein peroxidation thought to be crucial for hepatotoxicity [8,14].

In conclusion, similar results from both the present study and previous *in vivo* studies showed the effects of PER on redox systems, and the HepG2 cell line might be a useful and reliable non-animal alternative for clarifying mechanisms of

PER-related hepatotoxicity. In addition, further investigations are needed to determine the association of OS in HepG2 cells exposed to PER, which leads to lipid peroxidation, with different pathways, including cell death/apoptosis or autophagy, to be crucial in the cell/tissue damage related to pesticide toxicity.

Conflicts of Interest

There are no conflicts to declare

REFERENCES

1. Zhang SY, Ueyama J, Ito Y, Yanagiba Y, Okamura A, Kamijima M, Nakajima T: Permethrin may induce adult male mouse reproductive toxicity due to cis isomer not trans isomer. *Toxicology,* 248 (2-3): 136-141, 2008. DOI: 10.1016/j.tox.2008.03.018

2. Wang X, Martínez MA, Dai M, Chen D, Ares I, Romero A, Castellano V, Martínez M, Rodríguez JL, Martínez-Larrañaga MR, Anadon A, Yuan Z: Permethrin-induced oxidative stress and toxicity and metabolism. A review. *Environ Res,* 149, 86-104, 2016. DOI: 10.1016/j.envres.2016.05.003

3. U.S.EPA: Environmental Protection Agency. Reregistration eligibility decision (RED) on permethrin. EPA 738-R-09-306; US EPA: Washington, DC: 1-93, 2009.

4. Price RJ, Walters DG, Finch JM, Gabriel KL, Capen CC, Osimitz TG, Lake BG: A mode of action for induction of liver tumors by pyrethrins in the rat. *Toxicol Appl Pharmacol,* 218 (2): 186-195, 2007. DOI: 10.1016/j.taap.2006.11.004

5. Hakoi K, Cabral R, Hoshiya T, Hasegawa R, Shirai T, Ito N: Analysis of carcinogenic activity of some pesticides in a medium-term liver bioassay in the rat. *Teratog Carcinog Mutagen,* 12 (6): 269-276, 1992. DOI: 10.1002/tcm.1770120605

6. Imamura L, Hasegawa H, Kurashina K, Hamanishi A, Tabuchi A, Tsuda M: Repression of activity-dependent c-fos and brain-derived neurotrophic factor mRNA expression by pyrethroid insecticides accompanying a decrease in Ca^{2+} influx into neurons. *J Pharmacol Exp Ther,* 295 (3): 1175-1182, 2000.

7. Ishmael J, Litchfield MH: Chronic toxicity and carcinogenic evaluation of permethrin in rats and mice. *Fundam Appl Toxicol,* 11 (2): 308-322, 1988. DOI: 10.1016/0272-0590(88)90156-X

8. Gabbianelli R, Palan M, Flis DJ, Fedeli D, Nasuti C, Skarydova L, Ziolkowski W: Imbalance in redox system of rat liver following permethrin treatment in adolescence and neonatal age. *Xenobiotica,* 43 (12): 1103-1110, 2013. DOI: 10.3109/00498254.2013.796427

9. Abdou R, Sasaki K, Khalil W, Shah S, Murasawa Y, Shimoda M: Effects of several pyrethroids on hepatic cytochrome P450 activities in rats. *J Vet Med Sci,* 72 (4): 425-433, 2010. DOI: 10.1292/jvms.09-0347

10. Roma GC, Oliveira PRD, Bechara GH, Mathias MIC: Cytotoxic effects of permethrin on mouse liver and spleen cells. *Microsc Res Tech,* 75 (2): 229-238, 2012. DOI: 10.1002/jemt.21047

11. Nasuti C, Fattoretti P, Carloni M, Fedeli D, Ubaldi M, Ciccocioppo R, Gabbianelli R: Neonatal exposure to permethrin pesticide causes lifelong fear and spatial learning deficits and alters hippocampal morphology of synapses. *J Neurodev Disord,* 6:7, 2014. DOI: 10.1186/1866-1955-6-7

12. Jin Y, Chen R, Liu W, Fu Z: Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (Danio rerio). *Fish Shellfish Immunol,* 28 (5-6): 854-861, 2010. DOI: 10.1016/j.fsi.2010.02.009

13. Vadhana MSD, Nasuti C, Gabbianelli R: Purine bases oxidation and repair following permethrin insecticide treatment in rat heart cells. *Cardiovasc Toxicol,* 10 (3): 199-207, 2010. DOI: 10.1007/s12012-010-9079-6

14. Wang P, Xu MY, Liang YJ, Wang HP, Sun YJ, Long DX, Wu YJ: Subchronic toxicity of low dose propoxur, permethrin, and their combination on the redox status of rat liver. *Chem Biol Interact,* 272, 21-27, 2017. DOI: 10.1016/j.cbi.2017.04.023

15. Mozhdeganloo Z, Jafari AM, Koohi MK, Heidarpour M: Permethrin-induced oxidative damage in liver of rainbow trout *(Oncorhynchus mykiss)* and its attenuation by vitamin C. *Iran J Vet Res,* 17 (1): 31-35, 2016.

16. Roma GC, De Oliveira PR, Araujo AM, Bechara GH, Mathias MIC: Genotoxic and mutagenic effects of permethrin in mice: micronuclei analysis in peripheral blood erythrocytes. *Microsc Res Tech,* 75 (12): 1732-1736, 2012. DOI: 10.1002/jemt.22124

17. Hu F, Li L, Wang C, Zhang Q, Zhang X, Zhao M: Enantioselective induction of oxidative stress by permethrin in rat adrenal pheochromocytoma (PC12) cells. *Environ Toxicol Chem,* 29 (3): 683-690, 2010. DOI: 10.1002/etc.73

18. Donato MT, Tolosa L, Gómez-Lechón MJ: Culture and functional characterization of human hepatoma HepG2 cells. *Methods Mol Biol,* 1250, 77-93, 2015. DOI: 10.1007/978-1-4939-2074-7_5

19. Ramirez T, Strigun A, Verlohner A, Huener HA, Peter E, Herold M, Bordag N, Mellert W, Walk T, Spitzer M, Jiang X, Sperber S, Hofmann T, Hartung T, Kamp H, van Ravenzwaay B: Prediction of liver toxicity and mode of action using metabolomics *in vitro* in HepG2 cells. *Arch Toxicol,* 92 (2): 893-906, 2018. DOI: 10.1007/s00204-017-2079-6

20. Romero A, Ares I, Ramos E, Castellano V, Martínez M, Martínez-Larrañaga M, Anadón A, Martínez MA: Evidence for dose-additive effects of a type II pyrethroid mixture. *In vitro* assessment. *Environ Res,* 138, 58-66, 2015. DOI: 10.1016/j.envres.2015.02.008

21. Želježić D, Mladinić M, Žunec S, Vrdoljak AL, Kašuba V, Tariba B, Živković T, Marjanović AM, Pavičić I, Milić M, Rozgaj R, Kopjar N: Cytotoxic, genotoxic and biochemical markers of insecticide toxicity evaluated in human peripheral blood lymphocytes and an HepG2 cell line. *Food Chem Toxicol,* 96, 90-106, 2016. DOI: 10.1016/j.fct.2016.07.036

22. Mateos R, Goya L, Bravo L: Determination of malondialdehyde by liquid chromatography as the 2, 4-dinitrophenylhydrazone derivative: A marker for oxidative stress in cell cultures of human hepatoma HepG2. *J Chromatogr B Analyt Technol Biomed Life Sci,* 805 (1): 33-39, 2004. DOI: 10.1016/j.jchromb.2004.02.004

23. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254, 1976. DOI: 10.1006/abio.1976.9999

24. Rudzok S, Krejči S, Graebsch C, Herbarth O, Mueller A, Bauer M: Toxicity profiles of four metals and 17 xenobiotics in the human hepatoma cell line HepG2 and the protozoa *Tetrahymena pyriformis*-A comparison. *Environ Toxicol,* 26 (2): 171-186, 2011. DOI: 10.1002/tox.20541

25. Ayala A, Muñoz MF, Argüelles S: Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev,* 2014:360438, 2014. DOI: 10.1155/2014/360438

26. Zarkovic N, Cipak A, Jaganjac M, Borovic S, Zarkovic K: Pathophysiological relevance of aldehydic protein modifications. *J Proteomics,* 92, 239-247, 2013. DOI: 10.1016/j.jprot.2013.02.004

27. Abd El-Aal HAHM: Lipid peroxidation end-products as a key of oxidative stress: Effect of antioxidant on their production and transfer of free radicals. **In,** Catala A (Ed): Lipid Peroxidationed, InTech, 2012. DOI: 10.5772/45944

28. Ho E, Galougahi KK, Liu CC, Bhindi R, Figtree GA: Biological markers of oxidative stress: Applications to cardiovascular research and practice. *Redox Biol,* 1 (1): 483-491, 2013. DOI: 10.1016/j.redox.2013.07.006

29. Issam C, Zohra H, Monia Z, Hassen BC: Effects of dermal sub-chronic exposure of pubescent male rats to permethrin (PRMT) on the histological structures of genital tract, testosterone and lipoperoxidation. *Exp Toxicol Pathol,* 63 (4): 393-400, 2011. DOI: 10.1016/j.etp.2010.02.016

30. Grotto D, Maria LS, Valentini J, Paniz C, Schmitt G, Garcia SC, Pomblum VJ, Rocha JBT, Farina M: Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Quim Nova,* 32 (1): 169-174, 2009. DOI: 10.1590/S0100-40422009000100032

31. Lee YY, Kim HG, Jung HI, Shin YH, Hong SM, Park EH, Sa JH, Lim CJ: Activities of antioxidant and redox enzymes in human normal hepatic and hepatoma cell lines. *Mol Cells,* 14 (2): 305-311, 2002.

Kafkas Univ Vet Fak Derg
26 (2): 269-277, 2020
DOI: 10.9775/kvfd.2019.22932

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Effects of RNase III *rncS* Gene Deletion on Stress Response, Biofilm Formation and Virulence of *Listeria monocytogenes*

Lixia WANG [1,†,a] Mengfan QIAO [1,2,†,b] Qingling MENG [1,3,†,c] Jun QIAO [1,3,d] Yehui WU [1,e]
Jing GUO [1,f] Xifeng WANG [1,g] Jie LI [1,h] Xingxing ZHANG [3,i] Xuepeng CAI [4,j]

[†] *These authors contributed equally to this work*
[1] College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832003, CHINA
[2] School of Biological Engineering, Central South University, Changsha, Hunan, 410012, CHINA
[3] State Key Laboratory for Sheep Genetic Improvement and Healthy Production, Xinjiang Academy of Agricultural and Reclamation Science/Shihezi University, Shihezi, Xinjiang, 832000, CHINA
[4] State Key Lab of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu, 730046, CHINA
[a] ORCID: 0000-0001-8597-5998; [b] ORCID: 0000-0002-5432-0988; [c] ORCID: 0000-0002-1190-0114; [d] ORCID: 0000-0001-5210-4379
[e] ORCID: 0000-0003-0090-7035; [f] ORCID: 0000-0003-0021-4247; [g] ORCID: 0000-0001-8973-7399; [h] ORCID: 0000-0001-8423-2535
[i] ORCID: 0000-0001-8085-154X; [j] ORCID: 0000-0001-5725-1968

Article ID: KVFD-2019-22932 Received: 01.07.2019 Accepted: 15.12.2019 Published Online: 15.12.2019

How to Cite This Article

Wang L, Qiao M, Meng Q, Qiao J, Wu Y, Guo J, Wang X, Li J, Zhang X, Cai X: Effects of RNase III *rncS* gene deletion on stress response, biofilm formation and virulence of *Listeria monocytogenes*. *Kafkas Univ Vet Fak Derg,* 26 (2): 269-277, 2020. DOI: 10.9775/kvfd.2019.22932

Abstract

The ribonuclease III (RNase III) is an important enzyme system that regulates non-coding RNA (ncRNA) levels. In this study, LM-*ΔrncS* gene deletion strain was investigated by gene overlap extension PCR (SOE-PCR) and homologous recombination techniques. The environmental stress response, biofilm formation and virulence were determined and compared between the deletion strain LM-*ΔrncS* and the parental strain LM EGD-e. When compared with LM EGD-e, the adaptability of LM-*ΔrncS* was significantly reduced (P<0.05) under the stress of 30°C/42°C, pH 9, 5% NaCl, 3.8% ethanol and 0.1% H_2O_2. Biofilm formation ability of LM-*ΔrncS* was significantly lower (P<0.05) than that of LM EGD-e. In LM-*ΔrncS*, the transcription levels of ncRNA *SreA* and *SbrA* genes were significantly decreased (P<0.05). The adhesion rate and invasion rate of LM-*ΔrncS* in RAW264.7 cells were significantly lower (P<0.01) than those of LM EGD-e, and the survival and proliferation of LM-*ΔrncS* in RAW264.7 cells were also significantly decreased (P<0.05). Moreover, the transcription levels of *InlA, hly, prfA* and *SigmaB* gene were significantly lower (P<0.05) than those of LM EGD-e. LD_{50} of LD-*ΔrncS* in BALB/c mice was increased by 1.49 logarithmic orders, and the survival time of the mice was significantly prolonged when compared with LM EGD-e. In addition, the bacterial load in the liver and spleen was markedly decreased, and its pathological damage was also reduced. This study confirmed that RNase III RncS is involved in the regulation of environmental stress response, biofilm formation and virulence in LMd.

Keywords: *Listeria monocytogenes, RNase III rncS, Environmental stress response, Biofilm, Virulence*

Listeria monocytogenes'in RNase III rncS Geninin Silinmesinin Stres Tepkisi, Biyofilm Oluşumu ve Virülansı Üzerine Etkileri

Öz

Ribonükleaz III (RNase III), kodlama yapmayan RNA (ncRNA) seviyelerini düzenleyen önemli bir enzim sistemidir. Bu çalışmada, LM-*ΔrncS* gen silme suşu, gen örtüşme uzatma PCR (SOE-PCR) ve homolog rekombinasyon teknikleri ile incelendi. Çevresel stres tepkisi, biyofilm oluşumu ve virülans düzeyi belirlenerek silme suşu LM-*ΔrncS* ile ana suş LM EGD-e karşılaştırıldı. LM EGD-e ile karşılaştırıldığında, LM-*ΔrncS*'nin adapte olabilirliği 30°C/42°C, pH 9, %5 NaCl, %3.8 etanol ve %0.1 H_20_2 stresi altında önemli ölçüde azaldı (P<0.05). LM-*ΔrncS*'nin biyofilm oluşturma kabiliyeti, LM EGD-e'ninkinden anlamlı olarak daha düşüktü (P<0.05). LM-*ΔrncS*'de, ncRNA *SreA* ve *SbrA* genlerinin transkripsiyon seviyeleri anlamlı derecede azaldı (P<0.05). RAW264.7 hücrelerinde LM-*ΔrncS*'nin adezyon ve invazyon oranı LM EGD-e'ninkinden anlamlı olarak daha düşüktü (P<0.01) ve RAW264.7 hücrelerinde LM-*ΔrncS*'nin hayatta kalması ve çoğalması da önemli ölçüde azaldı (P<0.05). Bunun yanısıra, *InlA, hly, prfA* ve *SigmaB* genlerinin transkripsiyon seviyeleri LM EGD-e'ninkinden anlamlı derecede düşüktü (P<0.05). BALB/c farelerinde LD-*ΔrncS*'nin LD_{50}'si 1.49 logaritmik düzende arttırıldı ve farelerin hayatta kalma süresi LM EGD-e ile karşılaştırıldığında önemli ölçüde uzadı. Ek olarak, karaciğer ve dalaktaki bakteri yükü ve patolojik hasar da belirgin şekilde azaldı. Bu çalışma, RNase III RncS'nin LM'deki çevresel stres tepkisi, biyofilm oluşumu ve virülansın düzenlenmesinde rol oynadığını doğruladı.

Anahtar sözcükler: *Listeria monocytogenes, RNase III rncS, Çevresel stres yanıtı, Biyofilm, Virulans*

İletişim (Correspondence)
+86-993-2055036 Fax: 86-993-2055038
qj710625@126.com

INTRODUCTION

Listeria monocytogenes (LM) is a Gram-positive facultative anaerobic intracellular parasite that infects animals and humans, causing gastroenteritis, meningoencephalitis, abortion, sepsis and other symptoms in animals and immunocompromised populations such as elderly, newborn, and pregnant women, leading to high mortality and serious harm to animal husbandry production and food hygiene and safety [1,2]. As an important food-borne zoonotic pathogen, LM is widely found in nature and can survive in high-salt, hypertonic, low temperature, acidic and oxidative stress environments. It can form biofilms that are resistant to the external environment in animal foods and on the surface of processing containers [3]. Many studies have found that the strong viability and pathogenicity of LM are closely associated to its environmental stress factors and virulence factors [4,5]. To survive in stress conditions, LM has to modulate its transcriptions of related genes constantly to adapt to the different environments. Despite LM possesses many regulatory proteins to modulate gene expression, environmental stress factor Sigma B [4,5], positive regulatory factor PrfA and response regulator VirR [6,7] appear to play predominant roles in LM survival and infection. In recent years, studies have found that non-coding RNAs (ncRNAs) in LM can form complex regulatory networks with regulatory molecules such as PrfA, Sigma B and VirR, which precisely regulate the virulence and stress response-related genes of LM at transcription, post-transcription, and translational levels [6,8].

Ribonuclease III (RNase III) is a conserved RNase that cleaves a double-stranded RNA [9,10], which has been proved to be involved in the regulation of ncRNAs in bacteria [11,12]. However, the role and mechanism through which RNase III regulates the response of LM to environmental stress, biofilm formation and virulence is still unclear. In the present study, the main purpose was to explore the regulatory role of RNase III *rncS* in environmental stress response, biofilm formation and virulence of LM by constructing a RNase III *ΔrncS* gene deletion mutant of LM, which will provide an insight into the regulatory role of RNase III in environmental stress response and the pathogenesis of LM.

MATERIAL and METHODS

Primers

According to the genomic sequence of LM EGD-e registered in GenBank (accession number: AL591824), the specific primers of the *ΔrncS* deletion strain (R1-R6) and the qRT-PCR primers for the detection of virulence and biofilm formation-related genes were designed by Primer 5.0 software (Premier Inc, Canada). The cleavage sites of *Kpn* I and *Pst* I, and protective bases were added to the 5′ ends of the R1 and R4 primers, respectively. The information of these primers is shown in *Table 1*.

Table 1. *Primers used in the study*

Primer Names	Primer Sequences (5′→3′)
R1	GGGGTACCATGAATCAATGGGAAGAAT
R2	GTCCCGTTGAACAATTTCATATTTGTTAAATAGATA
R3	TATCTATTTAACAAATATGAAATTGTTCAACGGGAC
R4	TGCACTGCAGTTATCTGTGTATTAGTTTGTTTAT
R5	ACCACGGAGCGATTTAGC
R6	TTACGAGATTTGTTGGTTCA
SbrA F	CATCTAGATCCATACCCCTAAACTCCCT
SbrA R	AAAAAAAGAGCAGCACCCGAGAGTAC
SreA F	CAAGAAACGTCACTATAGTCACAAAT
SreA R	AAAAAAAGCCTTTCTGCTGATGAG
16s rRNA F	GAGCTAATCCCATAAAACTATTCTCA
16s rRNA R	ACCTTGTTACGACTTCACCCC
SigmaB F	CGCCGAATCAAAGAGTTAGG
SigmaB R	CTTTTTCCCATTTCCATTGCTTC
PrfA F	ACGGGAAGCTTGGCTCTATT
PrfA R	TGCGATGCCACTTGAATATC
hly F	TGCAAGTCCTAAGACGCCA
hly R	CACTGCATCTCCGTGGTATACTAA
inlA F	TGTGACTGGCGCTTTAATTG
inlA R	TCCAATAGTGACAGGTTGGCTA

Generation and Identification of LM-ΔrncS Deletion Strain

The LM EGD-e strain (a gift from W. Goebel of the University of Woodsburg, Germany) was inoculated in brain heart infusion (BHI) broth (Difco, USA) and cultured for 16 h at 37°C. The genomic DNA of the bacteria was extracted according to the instruction of the bacterial genomic DNA extraction kit (Omega, USA). Briefly, using the primer pairs R1-R2 and R3-R4, the upstream and downstream homologous arms were amplified using LM genomic DNA as the template, respectively. The fusion fragment of *rncS* deletion mutation was obtained by SOE-PCR and cloned into pMD19-T simple vector (TaKaRa, Japan) to generate the recombinant plasmid pMD19-T-*ΔrncS*. The pMD19-T-*ΔrncS* and pKSV7 plasmids were digested with *Kpn* I and *Pst* I (TaKaRa, Japan), respectively, and the digested target fragment was ligated to the pKSV7 vector by T4 DNA ligase (TaKaRa, Japan) to generate a recombinant shuttle plasmid pKSV7-*ΔrncS*. Then, pKSV7-*ΔrncS* was electroporated (2.5 kv, 5.0 ms) into LM EGD-e competent cells, and the cells were inoculated on BHI plates containing chloramphenicol (15 μg/mL, Amresco, USA) and cultured. Positive clones were screened by PCR using primers P5-P6. Homologous recombination was performed using the positive clones at 42°C and in the presence of chloramphenicol (15 μg/mL, Amresco, USA). The recombinant strain was screened by PCR and sequencing, and was passed continuously for 20 generations to obtain LM-*ΔrncS* deletion strain.

Effect of rncS Gene Deletion on the Environmental Stress Response of LM

In brief, LM EGD-e and LM-*ΔrncS* were inoculated separately in 1.0 mL BHI liquid medium, cultured at 37°C, transferred to BHI medium (Difco, USA) at a ratio of 1:100 and cultured at 30°C, 37°C, and 42°C with shaking (200 r/min), respectively. Then $OD_{600\ nm}$ value was measured every 1.5 h and the bacterial growth curve was plotted. At the same time, the bacterial solution was inoculated in BHI medium containing 3.8% alcohol, 5% NaCl, 0.1% H_2O_2 at pH 4, 7, or 9. The $OD_{600\ nm}$ value was measured at different times and the growth curve was prepared. The experiment was repeated 3 times.

Effect of rncS Gene Deletion on the Biofilm Formation Ability of LM

Briefly, 200 μL of LM EGD-e and LM-*ΔrncS* bacterial culture were added to a 96-well microplate, and the biofilm was prepared using the method of crystal violet staining as described by Peng [13]. Each sample was divided into 3 groups, with 8 replicates in each group, and the $OD_{570\ nm}$ value was determined by enzyme-linked detector (BIOTEK, USA). Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA). The morphological structure of the biofilm was observed and photographed under an inverted microscope (LEICA, Germany).

Determination of Cell Adhesion, Invasion and Intracellular Survival and Proliferation

Mouse macrophage RAW264.7 was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, USA) in 6-well plates. The adhesion rate, invasive rate and intracellular bacteria number were determined by the method described by Peng [14]. The experiment was repeated three times.

Detection of Relative Transcription Levels of Biofilm Formation and Virulence-related Genes by qRT-PCR

The transcription levels of biofilm formation genes *(SreA* and *SbrA)* and virulence-related genes *(InlA, hly, prfA* and *SigmaB)* were determined using qRT-PCR according to the method described by Kun [15], respectively. Briefly, total RNA of LM EGD-e and LM-*ΔrncS* strains were extracted by Trizol (Invitrogen, USA) and reverse transcribed into cDNA using AMV reverse transcription kit (TaKaRa, Japan). The biofilm formation genes *(SreA* and *SbrA)* and virulence-related genes *(InlA, hly, prfA* and *SigmaB)* was quantified by qRT-PCR using a LightCycler 480 instrument (Roche, Switzerland). Each sample had 3 replicates, and each experiment was repeated 3 times. The relative transcript levels of biofilm formation genes *(SreA* and *SbrA)* and virulence-related genes *(InlA, hly, prfA* and *SigmaB)* genes were calculated according to the $2^{-\Delta\Delta CT}$ method using the *16s rRNA* as an internal control. The results were statistically analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA).

Determination of LD_{50} of LM in Mice

The LD_0 and LD_{100} of LM EGD-e and LM-*ΔrncS* in mice were respectively determined by the method described by Peng et al.[14]. Based on the above results, 100 8-week-old BALB/c mice were randomly divided into LM EGD-e group and LM-*ΔrncS* group. Each group consisted of 5 subgroups, with 10 mice in each subgroup. Each mouse received 0.5 mL bacteria culture at different dilutions by intraperitoneal injection. The mice were observed for 7 days. The mortality of the mice was calculated and the LD_{50} of LM EGD-e and LM-*ΔrncS* was determined by Karber method, respectively.

Determination of Mouse Survival Rate

Briefly, thirty 8-week-old BALB/c mice were randomly divided into 3 groups, with 10 mice in each group. Two groups were intraperitoneally injected with 0.5 mL LM EGD-e or LM-*ΔrncS* bacterial solution, and the third group was intraperitoneally injected with 0.5 mL phosphate buffered saline (PBS). The mental state changes of the mice were observed every day for 7 days. The mental state and death in each group were recorded, and the Kaplan-Meier survival curve was prepared.

Bacterial Load in Mouse Organs and Histopathological Analysis

Eight-week-old BALB/c mice were randomly divided into 3 groups, with 7 mice in each group. The three groups of mice were intraperitoneally injected with 0.5 mL of sub-lethal dose of LM EGD-e, LM-*ΔrncS* bacterial solution or PBS buffer. After injection, the liver, spleen and kidney of a mouse in each group were extracted and cultured in BHI medium for bacteria counting. The procedure was repeated 3 times. On the 5th day, the liver, spleen and kidney of a mouse in each group were removed and fixed with 4% formaldehyde solution. After the tissue sections were prepared, histopathological changes of mice tissues were analyzed after hematoxylin and eosin (HE) staining.

Statistical Analysis

All data were statistically analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, CA, USA). Continuous variables were compared using *t* test, and categorical variables were analyzed using chi-square test. Data were expressed as mean ± standard deviation (SD). Statistically, $P<0.05$ was considered significantly different, $P<0.01$ was considered extremely significant difference.

RESULTS

The PCR amplification and sequence analysis confirmed that the LM-*ΔrncS* deletion strain was successfully constructed and identified.

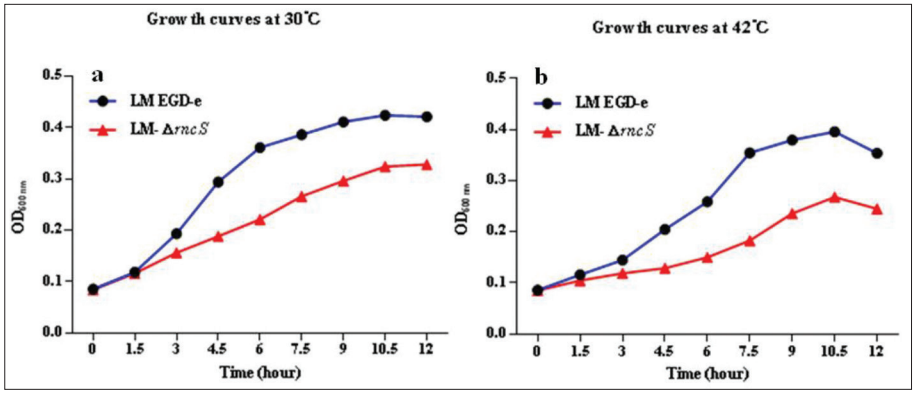


Fig 1. Growth curves of LM EGD-e and LM-△*rncS* at different temperatures. **a:** Growth curves of LM EGD-e and LM-△*rncS* at 30°C; **b:** Growth curves of LM EGD-e and LM-△*rncS* at 42°C

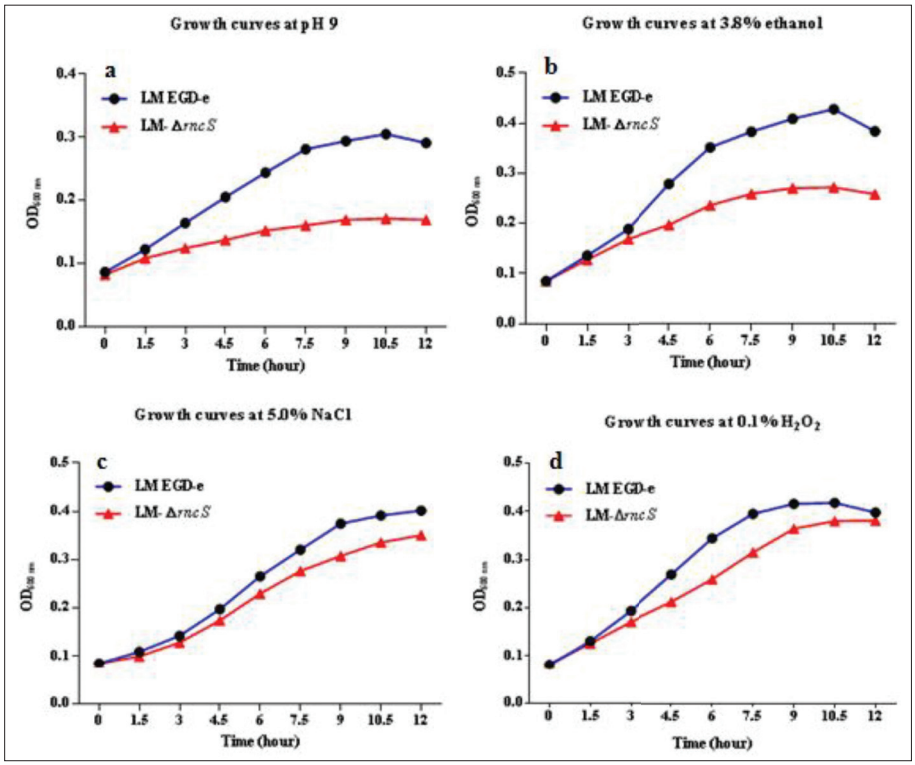


Fig 2. Growth curves of LM EGD-e and LM-△*rncS* at different stress conditions. **a:** Growth curves of LM EGD-e and LM-△*rncS* at pH 9; **b:** Growth curves of LM EGD-e and LM-△*rncS* at 3.8% ethanol; **c:** Growth curves of LM EGD-e and LM-△*rncS* at 5% NaCl; **d:** Growth curves of LM EGD-e and LM-△*rncS* at 0.1% H_2O_2

The growth curve showed that the growth of LM EGD-e and LM-*ΔrncS* was not significantly different at 37°C (P>0.05). However, at 30°C and 42°C, the growth difference between the two was significantly reduced after 4.5 h (P<0.01), indicating that the resistance of LM-*ΔrncS* to low temperature and high temperature was markedly decreased *(Fig. 1a,b)*.

At pH 4, both LM EGD-e and LM-*ΔrncS* were in a growth arrest state; at pH 7, the growth difference between the two bacterial strains was not significant (P>0.05); at pH 9, the growth of LM-*ΔrncS* was dramatically lower than that of LM EGD-e after being cultured for 3 h (P<0.05) *(Fig. 2a)*. Under the condition of 3.8% alcohol, the growth difference between the parental strain and the mutant strain after 4.5 h culture was extremely significant (P<0.01) *(Fig. 2b)*. Under 5% NaCl, the growth of the two strains was significantly different after 7.5 h culture (P<0.05) *(Fig. 2c)*. In the presence of 0.1% H_2O_2, the growth of LM-*ΔrncS* was significantly slower than LM EGD-e (P<0.01) after being cultured between 4.5 and 9 h *(Fig. 2d)*, revealing that the stress response of LM-*ΔrncS* under the conditions of alkali, high salt, alcohol and H_2O_2 is weaker than that of LM EGD-e.

At 24 h and 48 h, both LM EGD-e and LM-*ΔrncS* produced biofilms *(Fig. 3a,b,c,d)*, but the strength of LM-*ΔrncS* biofilm was significantly lower than that of LM EGD-e (P<0.05) *(Fig. 3e)*. The results of qRT-PCR showed that in the absence of *rncS* gene, the transcription levels of *SreA* and *SbrA* genes were decreased at 24 h and were significantly decreased significantly at 48 h (P<0.01) *(Fig. 4)*.

Cell infection assay showed that the adhesion rate and invasion rate of LM-*ΔrncS* in RAW264.7 cells were significantly lower than those of LM EGD-e (P<0.01) *(Fig. 5A,B)*. After infection, in different time periods, the amount of LM EGD-e and LM-*ΔrncS* was first decreased and then increased with time, and the amount of LM-*ΔrncS* was lower than that of

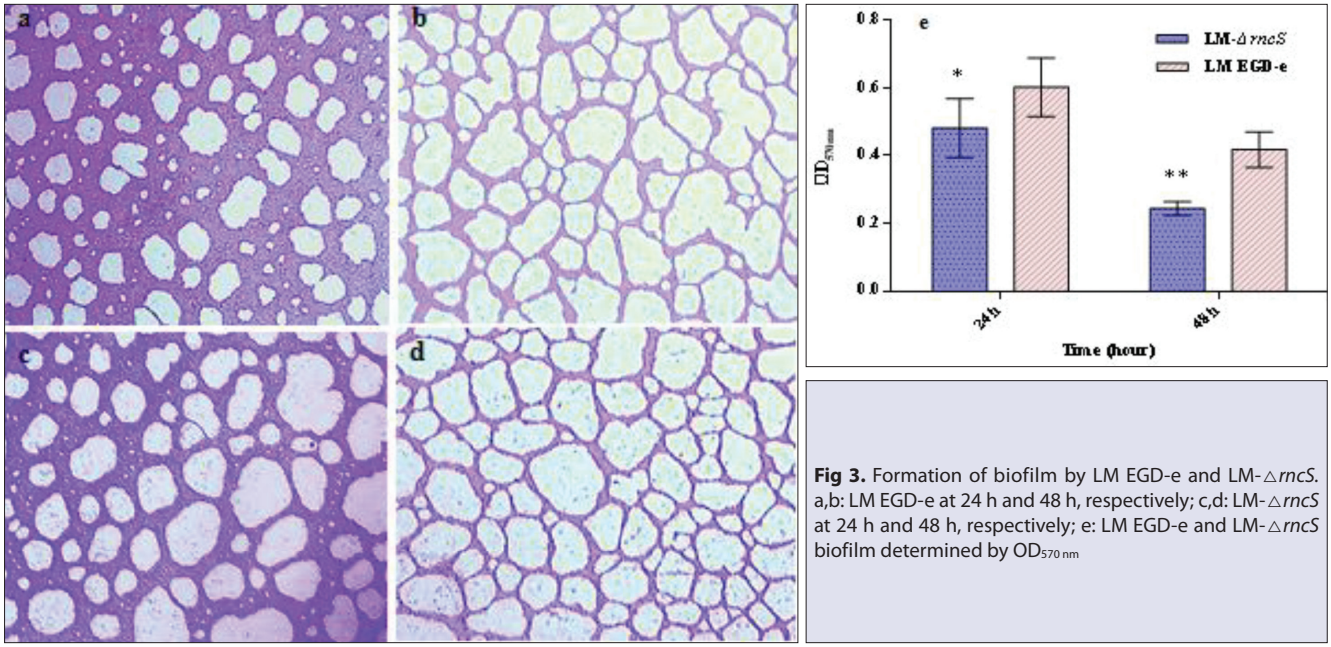


Fig 3. Formation of biofilm by LM EGD-e and LM-△*rncS*. a,b: LM EGD-e at 24 h and 48 h, respectively; c,d: LM-△*rncS* at 24 h and 48 h, respectively; e: LM EGD-e and LM-△*rncS* biofilm determined by $OD_{570\,nm}$

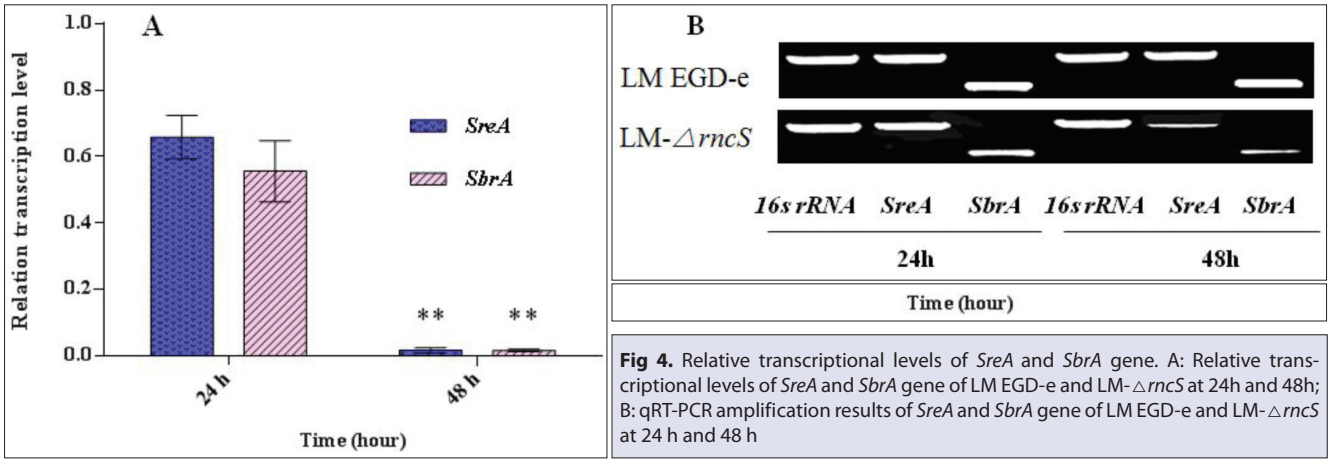


Fig 4. Relative transcriptional levels of *SreA* and *SbrA* gene. A: Relative transcriptional levels of *SreA* and *SbrA* gene of LM EGD-e and LM-△*rncS* at 24h and 48h; B: qRT-PCR amplification results of *SreA* and *SbrA* gene of LM EGD-e and LM-△*rncS* at 24 h and 48 h

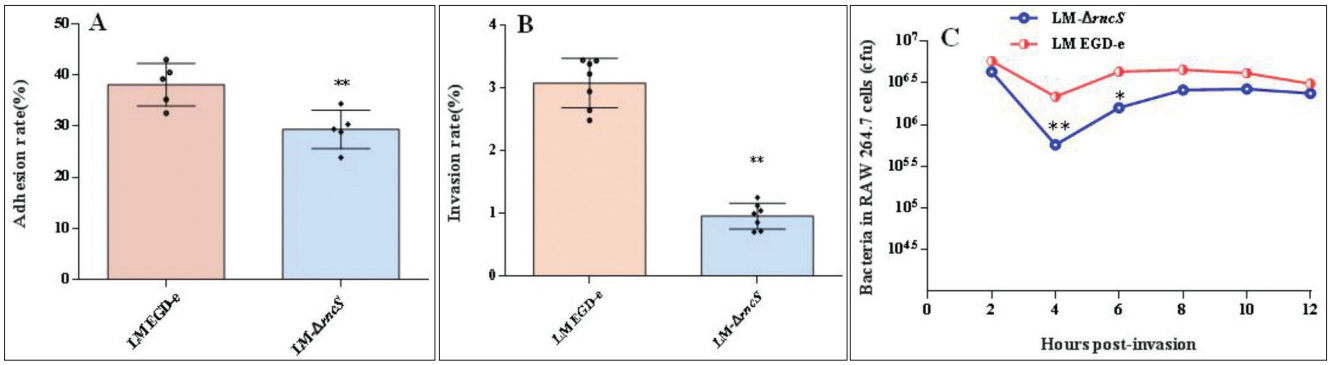


Fig 5. Invasion of LM EGD-e and LM-△*rncS* in RAW264.7 cells. A: LM EGD-e and LM-△*rncS* adhesion rate in RAW264.7 cells; B: LM EGD-e and LM-△*rncS* in RAW264.7 cell invasion rate; C: Bacterial numbers of LM EGD-e and LM-△*rncS* in the RAW264.7 cells after infection at various time

LM EGD-e throughout the whole time course *(Fig. 5C)*. At 2-4 h, the amount of both bacteria strains was decreased rapidly, and the decreasing trend of LM-*ΔrncS* was more obvious (P<0.01). At 4-6 h, both strains increased rapidly, and the difference was significant (P<0.05). Moreover, the transcription levels of *InlA*, *hly*, *prfA* and *SigmaB* gene were significantly lower than those of LM EGD-e (P<0.05) *(Fig. 6)*. These results of cell infection confirmed that *rncS* gene deletion reduced the survival and proliferation of LM EGD-e in RAW264.7 cells.

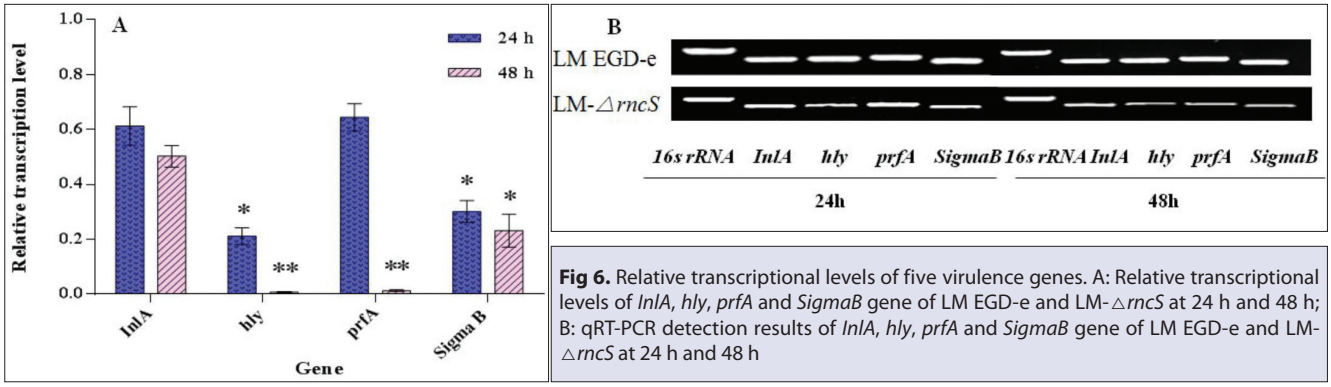
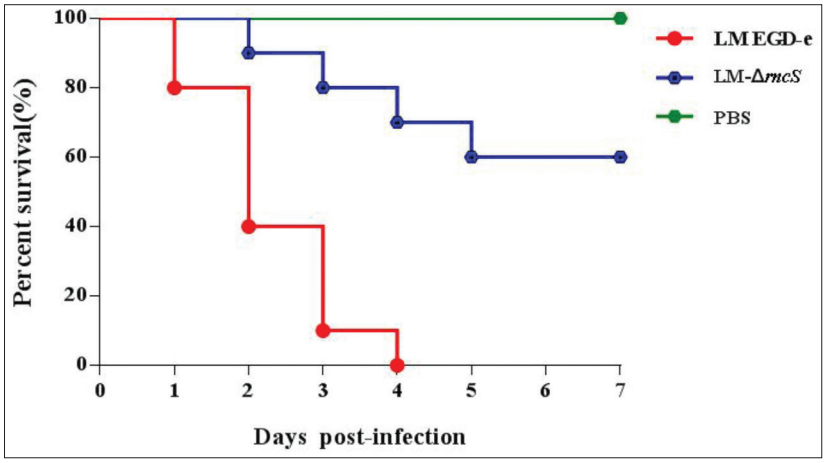


Fig 6. Relative transcriptional levels of five virulence genes. A: Relative transcriptional levels of *InlA*, *hly*, *prfA* and *SigmaB* gene of LM EGD-e and LM-△*rncS* at 24 h and 48 h; B: qRT-PCR detection results of *InlA*, *hly*, *prfA* and *SigmaB* gene of LM EGD-e and LM-△*rncS* at 24 h and 48 h

Fig 7. Survival curves of mice infected by intraperitoneal injection of LM EGD-e and LM-△*rncS*



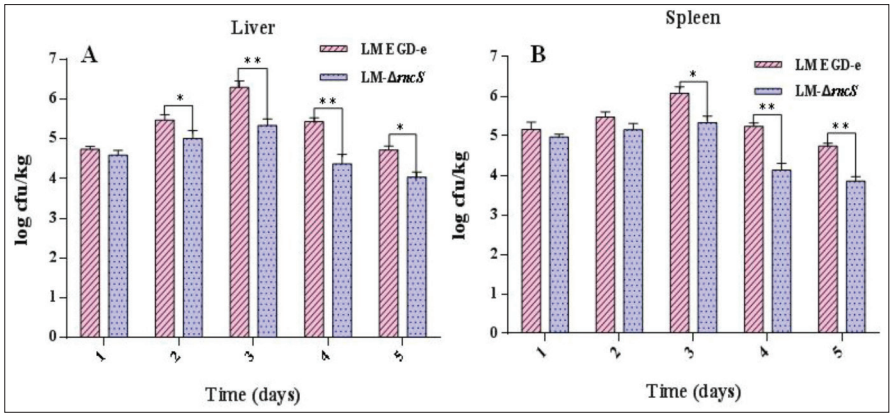


Fig 8. Bacterial load of LM EGD-e and LM-△*rncS* in the livers and spleens of mice infected by intraperitoneal infection. **A:** Bacterial numbers in the livers; **B:** Bacterial numbers in the spleens

Table 2. *LD_{50} measurement results of LM EGD-e and LM-ΔrncS*

Group	LM EGD-e			LM-ΔrncS		
	Dose/cfu	Mortality	LD_{50}	Dose/cfu	Mortality	LD_{50}
1	1.68×10^{6}	(10/10)	$10^{5.56}$	1.12×10^{9}	(10/10)	$10^{7.05}$
2	8.40×10^{5}	(9/10)		1.12×10^{8}	(8/10)	
3	4.20×10^{5}	(6/10)		1.12×10^{7}	(5/10)	
4	2.10×10^{5}	(2/10)		1.12×10^{6}	(2/10)	
5	1.05×10^{5}	(0/10)		1.12×10^{5}	(0/10)	

The LD_{50} of LM EGD-e and LM-*ΔrncS* in BALB/c mice were $10^{5.56}$ cfu and $10^{7.05}$ cfu, respectively *(Table 2)*. Compared with the LD_{50} of LM EGD-e, the LD_{50} of LM-*ΔrncS* increased by 1.49 logarithmic order, which indicated that the virulence of the bacteria was significantly decreased. Compared with LM EGD-e-infected mice, the survival time of LM-*ΔrncS*-infected mice was significantly prolonged *(Fig. 7)*, suggesting that the *rncS* gene has a regulatory effect on the virulence of LM.

After LM EGD-e and LM-*ΔrncS* infection in the liver and spleen, the bacterial load in the liver and spleen of LM-*ΔrncS*-infected mice was lower than that of LM EGD-e-infected mice at different time points of infection.

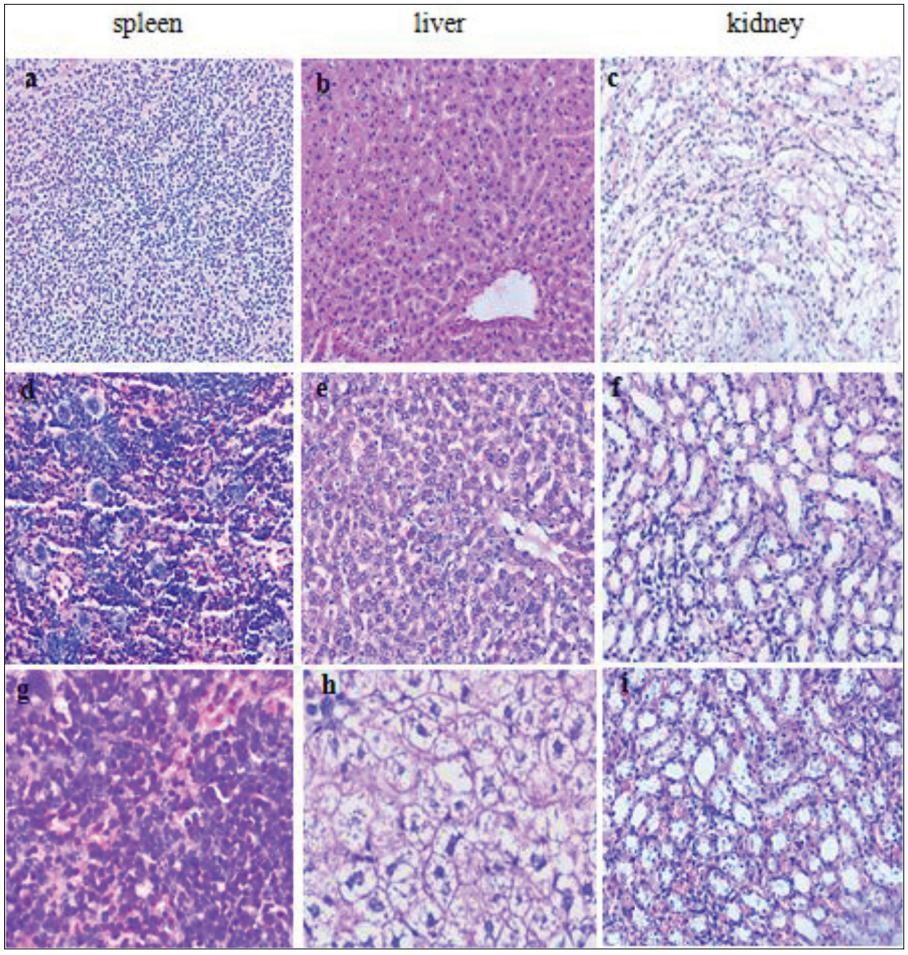


Fig 9. Histopathological examination of mice spleen, liver and kidney (HE×400). **a,b,c:** spleen, liver and kidney of normal mouse; **d,e,f:** spleen, liver and kidney of mice injected with LM-△*rncS*; **g,h,i:** spleen, liver and kidney of mice injected with LM EGD-e

In day 3-5, the bacterial load in the liver and spleen was significantly different between the two groups (P<0.05) *(Fig. 8A,B)*.

Histopathological analysis revealed that compared with the spleen *(Fig. 9a)*, liver *(Fig. 9b)* and kidney *(Fig. 9c)* of normal mice, the LM EGD-e-infected mice had partial hepatocyte necrosis, hepatic lobular inflammatory cell infiltration, the tissue structure was unclear and a reticular filamentous structure appeared *(Fig. 9h)*. Transparent degeneration and lymphoid tissue necrosis occurred in spleen reticular fibers, spleen nodules increased in size, intercellular structure was loose, and interstitial volume was widened *(Fig. 9g)*. Renal venular hemorrhage occurred, inflammatory cells appeared, and the interstitial volume was enlarged *(Fig. 9i)*. Compared with the LM EGD-e-infected mice, the pathological changes were obviously alleviated in LM-*ΔrncS*-infected mice *(Fig. 9d,e,f)*, indicating that *rncS* gene deletion reduced the virulence of LM.

DISCUSSION

Studies have found that RNaseIII is a highly conserved nuclease that can cleave double-stranded RNA and participates in the regulation of bacterial ncRNA, directly or indirectly affecting the adaptability of bacteria in stressful environments such as weak bases and oxidation [16-18]. Kim et al.[19] found that RNase III is involved in the regulation of low temperature stress response in *E. coli*. Roy and Chanfreau [20] confirmed that degradation of BDF2 mRNA by RNase III under hypertonic conditions resulted in a significant decrease in the growth of the yeast Δbdf1 deletion strain. So far, more than 200 ncRNAs have been found in LM [21]. These ncRNAs may be associated with various life activities such as acid-base tolerance, glucose metabolism and stress response of LM [22-24]. In this study, we confirmed that RNase III is involved in the regulation of stress response of LM to low temperature, high temperature, ethanol, high salt, alkaline and oxidative environment. However, which ncRNAs are required to mediate RNase III regulated environmental stress response of LM is still unclear. RNase III can indirectly affect the formation of biofilm by regulating the expression of some genes in bacteria. Saramago et al.[25] verified that in *R. typhimurium*, RNase III regulates the production of biofilms by regulating the mRNA level of a transcriptional regulator CsgD that regulates the expression of biofilm-associated matrix compounds and the expression of the flagellin *fljB* and *fliC* genes[26]. Kim et al.[27] revealed that RNase III can regulate the formation of *E. coli* biofilm through the RNase III-dependent pathway. Ruiz et al.[28] confirmed in *S. aureus*, the pairing of the biofilm repressor IcaR 3'-UTR with the 5'-UTR provides

a double-stranded RNA substrate for RNase III, which accelerates the decay of icaR mRNA and regulates the formation of biofilms. In addition, Zhao et al.[29] verified that RNase III indirectly regulates the formation of *Salmonella* typhimurium biofilm by catalytically cleaving the 3'UTR of the riboflavin synthase subunit RibE α mRNA to generate a novel ncRNA RibS. Lemon et al.[30] found that prfA plays a role in flagella-mediated movement and maturation of the biofilm after initial surface adhesion, whereas ncRNA *SreA* interacts with the 5'UTR of PrfA mRNA and reduces the stability of prfA transcript or prfA mRNA translation. In addition, *SreA* can also regulate the expression of AgrD [31] and indirectly regulate the adhesion process of biofilms [32]. Studies also revealed that σB regulates the formation of biofilm by regulating the expression of flagellin [33], while RNase III can cleave σB-dependent sRNA *SbrA* [34], suggesting that RNase III indirectly regulates the formation of biofilm. In this study, the phonotype analysis result demonstrated that the biofilm formation ability of LM-*ΔrncS* was significantly lower than that of LM EGD-e, and qRT-PCR further confirmed that the transcription levels of both *SreA* and *SbrA* genes were decreased, suggesting that RNase III may be indirectly involved in the regulation of biofilm formation through biofilm-associated ncRNA *SreA* and *SbrA*-mediated regulation.

Recent studies have also found that RNase III also plays an important regulatory role in the expression of bacterial virulence genes [35]. Darfeuille et al.[11] found in *E. coli* that RNase III mainly cleaves tisAB mRNA after IstR-1 sRNA binding. Furthermore, *in vivo* RyhB sRNA decay depends on RNase III after sRNA pairing with the 5'-UTR of its mRNA target [36]. Abidat revealed that RNase III can directly act on the virulence-related genes of *Salmonella*, and can also bind small non-coding antisense RNA molecules and mRNA target complexes to regulate the expression of virulence genes [25]. Bonnin and Bouloc [37] found that RNase III is involved in the regulation of the expression of cell adhesion factors in *S. aureus*. In this study, cell infection assay result confirmed that the adhesion, invasion and intracellular survival and proliferation of LM-*ΔrncS* in macrophages were significantly decreased. Meanwhile, the transcription levels of *InlA*, *hly*, *prfA* and *SigmaB* gene were significantly lower than those of LM EGD-e. In addition, the animal infection test further confirmed that the survival ability of LM-*ΔrncS* in the liver and spleen was significantly decreased, indicating that the *rncS* gene plays a regulatory role in the virulence of LM. However, which ncRNAs are involved in RNase III-mediated regulation of the virulence in LM require further study of transcriptomics of LM-*ΔrncS* deletion strain.

In conclusion, we for the first time confirmed that RNase III *rncS* is involved in the environmental stress response, biofilm formation and virulence in LM, which provide insights into the regulatory role of RNase III *rncS* in LM environmental adaptability and pathogenicity.

Acknowledgments

We thank the field staff who provided the samples for this study. This work was supported by the national key research and development program (No. 2016YFD0500900), Young and middle-aged leading science and technology innovation talents plan of Xinjiang Corps (No.2016BC001), the International Science & Technology Cooperation Program of China (No. 2014DFR31310), National Natural Science Foundation of China (31360596, 30960274), and Cooperation project of Urumqi-Chongqing in Science and technology (No. Y161220001).

Conflict of Interests Statement

The authors declare that they have no conflict of interest.

Ethical Approval

Ethical approval for this study was given by the Research and Ethical Committee of the Shihezi University.

REFERENCES

1. Mariscotti JF, Quereda JJ, Garcia-del Portillo F, Pucciarelli MG: The *Listeria monocytogenes* LPXTG surface protein Lmo1413 is an invasin with capacity to bind mucin. *Int J Med Microbiol*, 304 (3-4): 393-404, 2014. DOI: 10.1016/j.ijmm.2014.01.003

2. Quereda JJ, Pucciarelli MG: Deletion of the membrane protein Lmo0412 increases the virulence of *Listeria monocytogenes*. *Microbes Infect*, 16 (8): 623-632, 2014. DOI: 10.1016/j.micinf.2014.07.002

3. Lourenco A, de Las Heras A, Scortti M, Vazquez-Boland J, Frank JF, Brito L: Comparison of *Listeria monocytogenes* exoproteomes from biofilm and planktonic state: Lmo2504, a protein associated with biofilms. *Appl Environ Microbiol*, 79 (19): 6075-6082, 2013. DOI: 10.1128/AEM.01592-13

4. Guldimann C, Guariglia-Oropeza V, Harrand S, Kent D, Boor KJ, Wiedmann M: Stochastic and differential activation of sigma(B) and PrfA in *Listeria monocytogenes* at the single cell level under different environmental stress conditions. *Front Microbiol*, 8:348, 2017. DOI: 10.3389/fmicb.2017.00348

5. Dorey A, Marinho C, Piveteau P, O'Byrne C: Role and regulation of the stress activated sigma factor sigma B (σ^B) in the saprophytic and host-associated life stages of *Listeria monocytogenes*. *Adv Appl Microbiol*, 106, 1-48, 2019. DOI: 10.1016/bs.aambs.2018.11.001

6. Chakravarty S, Masse E: RNA-dependent regulation of virulence in pathogenic bacteria. *Front Cell Infect Microbiol*, 9:337, 2019. DOI: 10.3389/fcimb.2019.00337

7. Lebreton A, Cossart P: RNA and protein-mediated control of *Listeria monocytogenes* virulence gene expression. *RNA Biol*, 14 (5): 460-470, 2017. DOI: 10.1080/15476286.2016.1189069

8. Oliver HF, Orsi RH, Ponnala L, Keich U, Wang W, Sun Q, Cartinhour SW, Filiatrault MJ, Wiedmann M, Boor KJ: Deep RNA sequencing of *L. monocytogenes* reveals overlapping and extensive stationary phase and sigma B-dependent transcriptomes, including multiple highly transcribed noncoding RNAs. *BMC Genomics*, 10:641, 2009. DOI: 10.1186/1471-2164-10-641

9. Gagnon J, Lavoie M, Catala M, Malenfant F, Elela SA: Transcriptome wide annotation of eukaryotic RNase III reactivity and degradation signals. *Plos Genet*, 11 (2): e1005000, 2015. DOI: 10.1371/journal.pgen.1005000

10. MacRae IJ, Doudna JA: Ribonuclease revisited: structural insights into ribonuclease III family enzymes. *Curr Opin Struct Biol*, 17 (1): 138-145, 2007. DOI: 10.1016/j.sbi.2006.12.002

11. Darfeuille F, Unoson C, Vogel J, Wagner EG: An antisense RNA

inhibits translation by competing with standby ribosomes. *Mol Cell*, 26 (3): 381-392, 2007. DOI: 10.1016/j.molcel.2007.04.003

12. Wagner EGH, Altuvia S, Romby P: Antisense RNAs in bacteria and their genetic elements. *Adv Genet*, 46, 361-398, 2002. DOI: 10.1016/s0065-2660(02)46013-0

13. Peng YL, Meng QL, Qiao J, Xie K, Chen C, Liu TL, Hu ZX, Ma Y, Cai XP, Chen CF: The Regulatory roles of ncRNA Rli60 in adaptability of *Listeria monocytogenes* to environmental stress and biofilm formation. *Curr Microbiol*, 73 (1): 77-83, 2016. DOI: 10.1007/s00284-016-1028-6

14. Peng YL, Meng QL, Qiao J, Xie K, Chen C, Liu TL, Hu ZX, Ma Y, Cai XP, Chen CF: The roles of noncoding RNA *Rli60* in regulating the virulence of *Listeria monocytogenes*. *J Microbiol Immunol Infect*, 49 (4): 502-508, 2016. DOI: 10.1016/j.jmii.2014.08.017

15. Kun X, Qingling M, Qiao J, Yelong P, Tianli L, Cheng C, Yu M, Zhengxiang H, Xuepeng C, Chuangfu C: Impact of *rli87* gene deletion on response of *Listeria monocytogenes* to environmental stress. *FEMS Microbiol Lett*, 359 (1): 50-54, 2014. DOI: 10.1111/1574-6968.12561

16. Basineni SR, Madhugiri R, Kolmsee T, Hengge R, Klug G: The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target *rpoS* in *E. coli* is growth phase dependent. *RNA Biol*, 6 (5): 584-594, 2009. DOI: 10.4161/rna.6.5.10082

17. Caballero CJ, Menendez-Gil P, Catalan-Moreno A, Vergara-Irigaray M, Garcia B, Segura V, Irurzun N, Villanueva M, de los Mozos IR, Solano C, Lasa I, Toledo-Arana A: The regulon of the RNA chaperone CspA and its auto-regulation in *Staphylococcus aureus*. *Nucleic Acids Res*, 46 (3): 1345-1361, 2018. DOI: 10.1093/nar/gkx1284

18. Gordon GC, Cameron JC, Pfleger BF: Distinct and redundant functions of three homologs of RNase III in the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Nucleic Acids Res*, 46 (4): 1984-1997, 2018. DOI: 10.1093/nar/gky041

19. Kim KS, Manasherob R, Cohen SN: YmdB: A stress-responsive ribonuclease-binding regulator of *E. coli* RNase III activity. *Genes Dev*, 22, 3497-3508, 2008. DOI: 10.1101/gad.1729508

20. Roy K, Chanfreau G: Stress-induced nuclear RNA degradation pathways regulate yeast bromodomain factor 2 to promote cell survival. *Plos Genet*, 10 (9): e1004661, 2014. DOI: 10.1371/journal.pgen.1004661

21. Sievers S, Lund A, Menendez-Gil P, Nielsen A, Mollerup MS, Nielsen SL, Larsson PB, Borch-Jensen J, Johansson J, Kallipolitis BH: The multicopy sRNA LhrC controls expression of the oligopeptide-binding protein OppA in *Listeria monocytogenes*. *RNA Biol*, 12 (9): 985-997, 2015. DOI: 10.1080/15476286.2015.1071011

22. Opdyke JA, Kang JG, Storz G: GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. *J Bacteriol*, 186 (20): 6698-6705, 2004. DOI: 10.1128/JB.186.20.6698-6705.2004

23. Vanderpool CK, Gottesman S: Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. *Mol Microbiol*, 54 (4): 1076-1089, 2004. DOI: 10.1111/j.1365-2958.2004.04348.x

24. Valentin-Hansen P, Johansen J, Rasmussen AA: Small RNAs controlling outer membrane porins. *Curr Opin Microbiol*, 10 (2): 152-155, 2007. DOI: 10.1016/j.mib.2007.03.001

25. Saramago M, Domingues S, Viegas SC, Arraiano CM: Biofilm formation and antibiotic resistance in *Salmonella Typhimurium* are affected by different ribonucleases. *J Microbiol Biotechnol*, 24 (1): 8-12, 2014. DOI: 10.4014/jmb.1309.09046

26. Viegas SC, Mil-Homens D, Fialho AM, Arraiano CM: The virulence of *Salmonella enterica Serovar Typhimurium* in the insect model *Galleria mellonella* is impaired by mutations in RNase E and RNase III. *Appl Environ Microbiol*, 79 (19): 6124-6133, 2013. DOI: 10.1128/AEM.02044-13

27. Kim T, Lee J, Kim KS: *Escherichia coli* YmdB regulates biofilm formation independently of its role as an RNase III modulator. *BMC Microbiol*, 13:266, 2013. DOI: 10.1186/1471-2180-13-266

28. Ruiz de los Mozos I, Vergara-Irigaray M, Segura V, Villanueva M, Bitarte N, Saramago M, Domingues S, Arraiano CM, Fechter P, Romby P, Valle J, Solano C, Lasa I, Toledo-Arana A: Base pairing interaction between 5'- and 3'-UTRs controls icaR mRNA translation in *Staphylococcus aureus*. *Plos Genet*, 9 (12): e1004001, 2013. DOI: 10.1371/journal.pgen.1004001

29. Zhao X, Liu R, Tang H, Osei-Adjei G, Xu S, Zhang Y, Huang X: A 3' UTR-derived non-coding RNA RibS increases expression of cfa and promotes biofilm formation of *Salmonella enterica serovar Typhi*. *Res Microbiol*, 169 (6): 279-288, 2018. DOI: 10.1016/j.resmic.2018.04.007

30. Lemon KP, Freitag NE, Kolter R: The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. *J Bacteriol*, 192 (15): 3969-3976, 2010. DOI: 10.1128/JB.00179-10

31. Loh E, Dussurget O, Gripenland J, Vaitkevicius K, Tiensuu T, Mandin P, Repoila F, Buchrieser C, Cossart P, Johansson J: A trans-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. *Cell*, 139 (4): 770-779, 2009. DOI: 10.1016/j.cell.2009.08.046

32. Rieu A, Weidmann S, Garmyn D, Piveteau P, Guzzo J: Agr system of *Listeria monocytogenes* EGD-e: Role in adherence and differential expression pattern. *Appl Environ Microbiol*, 73 (19): 6125-6133, 2007. DOI: 10.1128/AEM.00608-07

33. Raengpradub S, Wiedmann M, Boor KJ: Comparative analysis of the σ^B-dependent stress responses in *Listeria monocytogenes* and *Listeria innocua* strains exposed to selected stress conditions. *Appl Environ Microbiol*, 74 (1): 158-171, 2008. DOI: 10.1128/AEM.00951-07

34. Nielsen JS, Olsen AS, Bonde M, Valentin-Hansen P, Kallipolitis BH: Identification of a σ^B -dependent small noncoding RNA in *Listeria monocytogenes*. *J Bacteriol*, 190 (18): 6264-6270, 2008. DOI: 10.1128/JB.00740-08

35. Matos RG, Casinhas J, Barria C, dos Santos RF, Silva IJ, Arraiano CM: The Role of ribonucleases and sRNAs in the virulence of foodborne pathogens. *Front Microbiol*, 8:910, 2017. DOI: 10.3389/fmicb.2017.00910

36. Afonyushkin T, Vecerek B, Moll I, Blasi U, Kaberdin VR: Both RNase E and RNase III control the stability of sodB mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res*, 33 (5): 1678-1689, 2005. DOI: 10.1093/nar/gki313

37. Bonnin RA, Bouloc P: RNA degradation in *Staphylococcus aureus*: Diversity of ribonucleases and their impact. *Int J Genomics*, 2015:395753, 2015. DOI: 10.1155/2015/395753

Kafkas Univ Vet Fak Derg
26 (2): 279-285, 2020
DOI: 10.9775/kvfd.2019.22936

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Hepatoprotective Activity of Silymarin in Combination with Clorsulon Against *Fasciola hepatica* in Naturally Infected Sheep

Mehrzad AFLATOUNI [1,a] Negar PANAHI [1,b] Pejman MORTAZAVI [2,c]
Bahar SHEMSHADI [2,d] Shapour KAKOOLAKI [3,e]

[1] Department of Veterinary Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, IRAN
[2] Department of Veterinary Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, IRAN
[3] Agricultural Research Education and Extension Organization (AREEO), Iranian Fisheries Science Research Institute (IFSRI), Tehran, IRAN
[a] ORCID: 0000-0001-6035-5434; [b] ORCID: 0000-0003-2261-3388; [c] ORCID: 0000-0002-2795-5371; [d] ORCID: 0000-0001-8996-3062
[e] ORCID: 0000-0003-2108-6985

Article ID: KVFD-2019-22936 Received: 30.06.2019 Accepted: 07.11.2019 Published Online: 08.11.2019

How to Cite This Article

Aflatouni M, Panahi N, Mortazavi P, Shemshadi B, Kakoolaki S: Hepatoprotective activity of silymarin in combination with clorsulon against *Fasciola hepatica* in naturally infected sheep. *Kafkas Univ Vet Fak Derg,* 26 (2): 279-285, 2020. DOI: 10.9775/kvfd.2019.22936

Abstract

Fasciolosis is a parasitic infection with socioeconomic implications that causes essential health problems in animals throughout the world. The current study intended to evaluate the hepatoprotective activity of silymarin alone and in combination with clorsulon in sheep naturally infected with *Fasciola hepatica*. For this purpose, a total of 40 sheep (Sangsari breed) were divided into five groups as follows: 1) the healthy sheep without treatment (the negative control), 2) the infected sheep without treatment (the positive control), 3) the infected sheep treated with silymarin (280 mg orally from the day 1 to 14), 4) the infected sheep treated with clorsulon (7.5 mg/kg orally on the days 5, 10, and 15), and 5) the infected sheep treated with silymarin + clorsulon. The assessed serum parameters included total bilirubin, total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT). Additionally, histopathological changes in naturally *F. hepatica*-infected sheep and controls were investigated using light microscopy. Biochemical analysis showed significantly lower levels of AST, ALT, ALP, GGT, bilirubin, protein, and albumin in infected animals than in the healthy group ($P<0.05$). Silymarin plus clorsulon treatment showed a remarkable improvement in both biochemical and histopathological parameters ($P<0.05$). In conclusion, the results of the biochemical analysis were in line with the pathological findings showing that silymarin, in combination with clorsulon, was superior to each compound alone in terms of hepatoprotective activity.

Keywords: *Fasciola hepatica, Sheep, Silymarin, Hepatoprotective, Biochemical parameters, Clorsulon*

Fasciola hepatica İle Doğal Enfekte Koyunlarda Silymarinin Klorsulon İle Kombinasyonunun Hepatoprotektif Aktivitesi

Öz

Fasciolosis, tüm dünyada hayvanlarda temel sağlık sorunlarına neden olan ve sosyoekonomik etkilere sahip paraziter bir enfeksiyondur. Bu çalışma, *Fasciola hepatica* ile doğal olarak enfekte olmuş koyunlarda silymarinin tek başına ve klorsulon ile kombinasyon halinde kullanımının hepatoprotektif aktivitesini değerlendirmeyi amaçlamıştır. Bu amaçla toplam 40 koyun (Sangsari ırkı) beş gruba ayrıldı: 1) tedavi görmeyen sağlıklı koyunlar (negatif kontrol), 2) tedavi görmemiş enfekte koyunlar (pozitif kontrol), 3) silymarin ile tedavi edilen enfekte koyunlar (1. günden 14. güne kadar oral yoldan 280 mg), 4) klorsulon ile tedavi edilen enfekte koyunlar (5, 10 ve 15 günlerde ağızdan 7.5 mg/kg) ve 5) silymarin + klorsulon ile tedavi edilen enfekte koyunlar. Serum parametreleri olarak total bilirubin, toplam protein, albümin, alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), alkalin fosfataz (ALP) ve gama-glutamil transferaz (GGT) incelendi. Bunun yanı sıra, doğal olarak *F. hepatica* ile enfekte ve kontrol grubundaki koyunlarda histopatolojik değişiklikler ışık mikroskobu kullanılarak incelendi. Analiz sonuçları, enfekte hayvanlarda AST, ALT, ALP, GGT, bilirubin, protein ve albümin düzeylerinin sağlıklı gruba göre anlamlı derecede düşük olduğunu gösterdi ($P<0.05$). Silymarin ve klorsulon kombinasyonu ile yapılan tedavide, hem biyokimyasal hem de histopatolojik parametrelerde kayda değer bir iyileşme gözlendi ($P<0.05$). Sonuç olarak, biyokimyasal analiz sonuçları, patolojik bulgularla uyumlu olarak, silimarinin klorsulon ile kombinasyonuyla yapılan tedavinin, hepatoprotektif aktivite açısından diğer tedavilerden daha üstün olduğunu gösterdi.

Anahtar sözcükler: *Fasciola hepatica, Koyun, Silymarin, Hepatoprotektif, Biyokimyasal parametreler, Klorsulon*

İletişim (Correspondence)
+98 912 2137206
n.panahi@srbiau.ac.ir

INTRODUCTION

Fasciolosis is a parasitic disease in domestic animals and humans mainly caused by a hepatic trematode parasite, *F. hepatica*. The parasite usually localized in the liver, gallbladder, and bile ducts of the host [1]. This infection has particular implications as it reduces meat and milk production, quantity and quality of wool, and fertility. One of the most effective flukicide compounds among anthelmintic drugs is clorsulon that belongs to sulphonamides [1-3]. Based on fecal egg count or postmortem studies, clorsulon is mainly effective against mature flukes via subcutaneous injection or orally [4].

Although chemical compounds (clorsulon, triclabendazole, and albendazole) [5] can facilitate parasite control, traces of hepatic injuries or pathological sequelae of the parasite remain in the liver of the host, making such drugs an inappropriate choice for medical application [6,7]. The use of different compounds in combination is an excellent strategy to act against both parasite and its pathological sequelae. Due to its unique hepatoprotective, antioxidant, and anti-inflammatory activities, silymarin is currently at the core of many biological innovations in clinical medicine. It is a mixture of phenylpropanoid and flavonoid derived from the extract of *Silybum marianum* (Milk thistle) [8]. The use of this plant in liver disease and the gastrointestinal problem describes in ancient medical books [9]. In folk medicine, in many countries, *S. marianum* (L.) is used for the treatment of gallbladder and liver disorders (jaundice, cirrhosis, and hepatitis) [10] and protection against environmental and chemical liver poisoning [11]. Silymarin is widely used as a nutritional supplement or therapeutic agent in liver disease without serious side-effects in both animals and humans [12,13].

The current study designed to evaluate the hepatoprotective activity of silymarin alone and in combination with clorsulon in sheep infected with *F. hepatica* by measuring some hepatic biochemical parameters (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin, albumin, and total protein). Moreover, the hepatoprotective activity of the compounds was assessed using histopathological analysis.

MATERIAL and METHODS

Drugs and Dosage

Aqueous suspension of silymarin (Livergol®; Goldaru, Iran) 280 mg was given from day 1 to 14 [14] and clorsulon (Triveni Chemicals, India) 7.5 mg/kg/day administered orally on the days 5, 10, and 15.

Experimental Design

All experiments were directed in agreement with the Guide for the Care and Use of Laboratory Animals in Research, approved by the University.

Forty male and female Sangsari sheep, 3-5-year-old, weighing 30-55 kg, were assigned to five groups of eight sheep. Before starting the experiment, 100 sheep were inspected for the presence of *F. hepatica* based on the egg count of fecal samples [15]. The fecal samples were collected directly from the rectum and preserved in a plastic bottle containing a 10% formalin solution [16,17]. Thirty-two *F. hepatica*-infected sheep selected by the sedimentation technique allocated to four accidental groups. A healthy animal group was also used for better comparing the hepatoprotective effects of the compounds. Groups were designated as follows: group 1 (H), the healthy sheep without treatment (the negative control, n=8); group 2 (F), the infected sheep without treatment (the positive control, n=8); group 3 (F+S), the infected sheep with silymarin treatment (280 mg orally from the day 1 to 14, n=8); group 4 (F+CL) (the infected sheep with clorsulon treatment (7.5 mg/kg orally on the days 5, 10, and 15, n=8); and group 5 (F+S+CL), the infected sheep with clorsulon plus silymarin treatment (n = 8).

Blood Samples and Biochemical Analysis

Blood samples from the external jugular vein were collected on days 0, 7, 14, and 21 and kept at +4°C for further biochemical analysis. For serum separation, blood tubes centrifuged at 3000 rpm for 10 min. Due to the poor stability of enzymes and bilirubin in the serum, the samples analyzed within six h. Liver enzymatic activities of AST, GGT, ALP, and total bilirubin, and total protein measured according to standard procedures using a BT-1500 automatic analyzer.

Histopathological Analysis

Before the animals slaughtered, the transversal sections of all liver lobes were collected, fixed in buffered formaldehyde solution, embedded in paraffin, and sent to a histopathological laboratory. Five-micrometer sections were stained based on the hematoxylin-eosin (H & E) technique and examined under light microscopy. The levels of inflammation [18], bile duct hyperplasia, necrosis, and fibrosis were determined in 5 to 10 histological sections per animal, randomly chosen. Two blinded observers performed all evaluations. The score of lesions were distinguished as follows: Necrosis: 0, none; 1, focal necrosis less than 25%; 2, focal necrosis 25-50%; 3, zonal necrosis 50-75%; 4, massive necrosis. Inflammation: 0, none; 1, focal inflammation less than 25%; 2, focal inflammation between 25-50%; 3, zonal inflammation; 4, extensive inflammation. Fibrosis: 0, none; 1, periportal fibrosis; 2, portal expansion; 3, septal development; 4, global septal fibrosis. Hyperplasia of the bile duct: 0, none; 1, mild hyperplasia less than 25%; 2, moderate hyperplasia 25-50% lesions; 3, severe hyperplasia between 50-75% lesions; 4, extensive hyperplasia [19].

Statistical Analysis

Statistical comparisons and graphs performed with GraphPad Prism software using the one-way and two-way analysis of variance (ANOVA) tests. P-values of less than 0.05 were measured statistically significant.

RESULTS

Biochemical Parameters

In the present study, the mean serum levels of ALT, ALP, AST, and GGT were significantly higher in the infected sheep groups than in the healthy group before treatment. The mean levels of ALT, ALP, AST, and GGT in F. hepatica-infected sheep decreased following the administration of the combination treatment with silymarin and clorsulon after 21 days *(Table 1)*. The hepatoprotective effect of silymarin was also remarkable on the level of these enzymes at different intervals, so that the mean levels of these enzymes decreased on the day 21 in the silymarin-treated group compared to the day 1 ($P<0.05$; *Table 1)*.

As shown in *Table 1*, the total protein and albumin levels were significantly lower in the infected groups than in the healthy group on day 1 of the study. Lower levels of total protein and albumin are also other indicators of liver disorder or injury. As shown in *Table 1*, the primary levels of these proteins were significantly lower in the control group than in the healthy group. Comparison of before and after treatment (the day 1, 7, 14, 21) by silymarin with/without clorsulon significantly increased the total protein

Table 1. *Time variations of biochemical parameters in F. hepatica-infected sheep treated with Silymarin, Clorsulon, and their combination*

Group	Time Interval (d)	Group 1 (H)	Group 2 (F)	Group 3 (F+S)	Group 4 (F+CL)	Group 5 (F+S+CL)
Total bilirubin	1	0.13±0.04 $^{a\#}$	2.57±0.08 $^{b\#}$	2.61±0.21 $^{b\#}$	2.81±0.14 $^{b\#}$	2.87±0.10 $^{b\#}$
	7	0.16±0.03 $^{a\#}$	2.57±0.086 $^{b\#}$	2.52±0.10 $^{b\#*}$	2.24±0.13 $^{b\#}$	2.11±0.15 $^{b\#}$
	14	0.15±0.03 $^{a\#}$	2.89±0.1 $^{b\#}$	2.45±0.13 ^{c*}	1.88±0.21 $^{c\#}$	0.97±0.31 ^{d*}
	21	0.15±0.02 $^{a\#}$	3.52±0.18 $^{b\#}$	2.34±0.01 ^{c*}	1.13±0.12 ^{c*}	0.18±0.07 $^{a\wedge}$
Albumin	1	6.52±1.01 ^{a}	4.6±0.7 $^{b\#*}$	4.8±0.3 $^{b\#}$	4.4±0.3 $^{b\#*}$	3.9±0.5 $^{b\#}$
	7	6.25±1.05 ^{a}	4.5±0.4 ^{bx}	4.6±0.3 $^{b\#}$	4.7±0.3 $^{b\#*}$	4.6±0.5 $^{b\#*}$
	14	6.25±0.9 ^{a}	3.6±0.5 $^{b*x\wedge}$	4.3±0.2 $^{bc\#}$	5.2±0.7 $^{ac\#*}$	5.8±0.45 ^{b*x}
	21	6.12±1.01 ^{a}	2.9±0.3 $^{b\#\wedge}$	3.9±0.2 $^{c\#}$	6.1±0.6 ^{a}	6.7±0.14 ^{cx}
Total protein	1	8.08±0.3 ^{a}	5.32±1.17 $^{b\#}$	5.2±0.8 $^{b\#}$	5.5±1.0 $^{b\#}$	5.5±0.5 $^{b\#}$
	7	8.9±0.19 ^{a}	5.2±1.1 $^{b\#}$	5.1±0.6 $^{b\#}$	5.4±1.01 $^{b\#}$	6.17±0.3 $^{b\#*}$
	14	8.0±0.3 ^{a}	4.9±0.8 $^{b\#}$	4.9±0.7 $^{b\#}$	6.75±1.6 $^{a\#*}$	7.4±0.7 ^{a*}
	21	7.92±0.6 ^{a}	4.4±0.4 ^{b}	4.17±0.8 ^{b}	7.8±2.1 ^{a*}	9.1±1.2 ^{a}
AST	1	68±2.5 $^{a\#}$	177±0.6 $^{b\#}$	194±4.5 $^{b\#*}$	184±3.4 $^{b\#}$	176±3.1 $^{b\#}$
	7	63±1.9 $^{a\#}$	185±1.6 $^{b\#}$	202±3.1 $^{b\#}$	158±2.7 $^{b\#}$	173±3.4 $^{b\#}$
	14	67±2.4 $^{a\#}$	164±1.3 $^{b\#}$	159±2.7 $^{b\#*}$	158±3.1 $^{b\#}$	176±2.3 $^{b\#}$
	21	61±2.3 $^{d\#}$	176±0.4 $^{ac\#}$	131±2.3 ^{a*}	192±2.1 $^{c\#}$	75±3.7 ^{d*}
ALT	1	49±3.3 ^{a}	60±3.4 ^{a}	51±4.3 ^{a}	59±3.1 ^{a}	69±4.2 $^{a\#}$
	7	41±2.9 ^{a}	65±4.1 ^{b}	50±3.2 ^{ab}	56±4.1 ^{b}	58±3.1 $^{a\#}$
	14	40±4.2 ^{a}	68±3.7 ^{b}	49±3.3 ^{a}	51±4.2 ^{a}	49±3.4 $^{a\#*}$
	21	46±3.6 ^{a}	72±4.1 ^{b}	47±3.4 ^{a}	42±3.8 ^{ac}	37±2.9 ^{c*}
ALP	1	16±3.6 $^{a\#}$	324±12.1 $^{b\#}$	328±13.3 $^{b\#}$	361±13.9 $^{b\#}$	405±23.9 $^{b\#}$
	7	16±3.6 $^{a\#}$	392±15.7 $^{b\#}$	323±11.4 $^{b\#}$	311±13.4 $^{b\#}$	321±17.8 $^{b\#}$
	14	15±3.3 $^{a\#}$	408±14.5 $^{b\#}$	350±17.2 $^{bc\#}$	372±11.7 $^{b\#}$	273±13.2 $^{c\#}$
	21	15±2.7 $^{d\#}$	528±13.6 ^{a*}	415±16.6 ^{ab*}	227±10.2 ^{bc*}	141.7±12.5 ^{c*}
GGT	1	2.75±0.5 $^{a\#}$	26.5±8.2 $^{b\#}$	26.2±9.03 $^{b\#}$	30±12.1 $^{bc\#}$	38.5±19.1 $^{c\#}$
	7	2. 5±0.5 $^{a\#}$	30.5±11.8 $^{b\#}$	30.7±10.4 $^{b\#}$	26.2±11 $^{b\#}$	33.7±14.2 $^{b\#}$
	14	2.25±0.5 $^{a\#}$	37.3±10.1 $^{b\#*}$	35±10.1 $^{b\#}$	21.7±9.8 $^{bc\#}$	19.25±5.1 $^{c\#*}$
	21	2.25±0.5 $^{c\#}$	51.3±12.3 ^{a*}	43.75±3.07 $^{a\#}$	15.5±11.01 $^{b\#}$	10.75±2.2 ^{b*}

*Data are expressed as mean ± standard error of the mean (SEM). Means with superscripts a, b, c, and d along a row differ significantly at $P<0.05$; Means with superscripts #, *, ^, and x along a column differ significantly at $P<0.05$. Group 1 (H), the healthy sheep without treatment; group 2 (F), the infected sheep without treatment; group 3 (F+S), the infected sheep with silymarin treatment (280 mg orally from the day 1 to 14); group 4 (F+CL) the infected sheep with clorsulon treatment (7.5 mg/kg orally on the days 5, 10, and 15); and group 5 (F+S+CL), the infected sheep with clorsulon plus silymarin treatment*

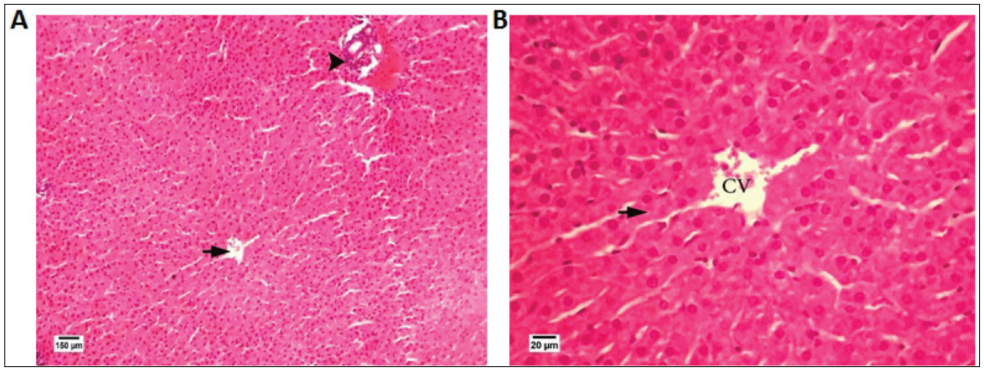


Fig 1. Liver tissue samples of sheep in the group 1 (the healthy group) show no pathological changes. *Arrowheads* point to A, central vein (H&E, 40X) and B, healthy hepatocytes (H&E, 400X)

Table 2. *The pathological findings of sheep liver in all the groups*

Parameter	Group 1 (H)	Group 2 (F)	Group 3 (F+S)	Group 4 (F+CL)	Group 5 (F+S+CL)
Necrosis	0	3.375±0.51 [B]	3.5±0.53 [B]	2.125±0.35 [B]	0.875±0.35 [AC]
Inflammation	0	3.5±0.53 [B]	3.25±0.46 [B]	2.375±0.51 [C]	0.87±0.35 [AC]
Fibrosis	0	3.37±0.51 [B]	3.25±0.46 [B]	2.25±0.43 [C]	0.75±0.46 [AC]
Bile duct hyperplasia	0	3.5±0.53 [B]	3.75±0.46 [B]	2.25±0.46 [C]	0.75±0.46 [AC]
Total lesion	0	13.75±1.28	13.75±0.7	9±0.75	3.25±0.1

[A] P<0.05 compared with the healthy group; [B] P<0.0001 compared with the healthy group; [C] P<0.0001 compared with the F. hepatica infected group. F and P-value for within and between factors are as follows: Row factor, F (3, 140) = 1.018, P = 0.3867. Column factor, F (4, 140) = 381.2, P<0.0001. Data are expressed as mean + SEM. Group 1 (H), the healthy sheep without treatment; group 2 (F), the infected sheep without treatment; group 3 (F+S), the infected sheep with silymarin treatment (280 mg orally from the day 1 to 14); group 4 (F+CL) the infected sheep with clorsulon treatment (7.5 mg/kg orally on the days 5, 10, and 15); and group 5 (F+S+CL), the infected sheep with clorsulon plus silymarin treatment. Lesion score: Necrosis: 0, none; 1, focal necrosis less than 25%; 2, focal necrosis between 25-50%; 3, zonal necrosis between 50-75%; 4, massive necrosis. Inflammation: 0, none; 1, focal inflammation less than 25%; 2, focal inflammation between 25-50%; 3, zonal inflammation; 4, extensive inflammation. Fibrosis: 0, none; 1, periportal fibrosis; 2, portal expansion; 3, septal development; 4, global septal fibrosis. Hyperplasia of the bile duct: 0, none; 1, mild hyperplasia less than 25%; 2, moderate hyperplasia between 25-50% lesions; 3, severe hyperplasia between 50-75%; 4, extensive hyperplasia

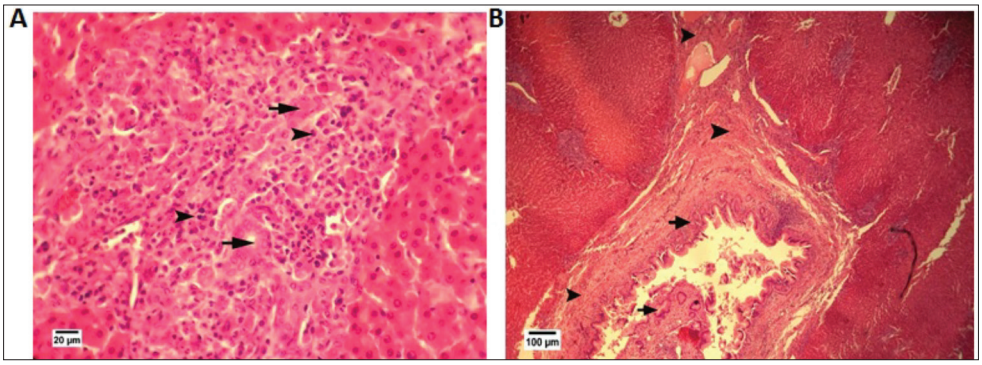


Fig 2. Liver tissue samples of sheep in the group 2 (no treatment) show pathological changes. *Arrowheads* point to immune cell infiltration, and *arrow* indicates fibrosis and bile duct hyperplasia (A, 400X &B, 100X)

and albumin levels, which is a significant difference in the combination of these two drugs.

In the present study, the serum level of total bilirubin was remarkably higher in the infected groups than in the healthy group. The increased concentration of total bilirubin is another indicator of hepatic injury. While clorsulon showed a moderate effect on decreasing total bilirubin, treatment with a combination of silymarin and clorsulon resulted in a significant decrease in the serum level of total bilirubin at the end of the experiment *(Table 1)*. The total bilirubin level was also significantly lower in the group treated with silymarin alone than in the infected untreated group (P<0.01).

Effects of Silymarin, Clorsulon, and Their Combination on Histopathological Parameters

Liver histopathology disclosed more inflammatory cell infiltration, fibrosis, necrosis, and bile duct hyperplasia in *F. hepatica*-infected sheep on day one than in the healthy group *(Fig. 1)*. As shown in *Fig. 1A,B*, the liver of group 1 (the healthy sheep with no treatment), had normal hepatocytes without any pathological lesions.

Fig. 2 shows the effects of *F. hepatica* parasites on the liver of the control group (without any treatment). The parasite caused massive necrosis, fibrosis, hepatitis, bile duct hyperplasia, and high rate of immune cell infiltration into

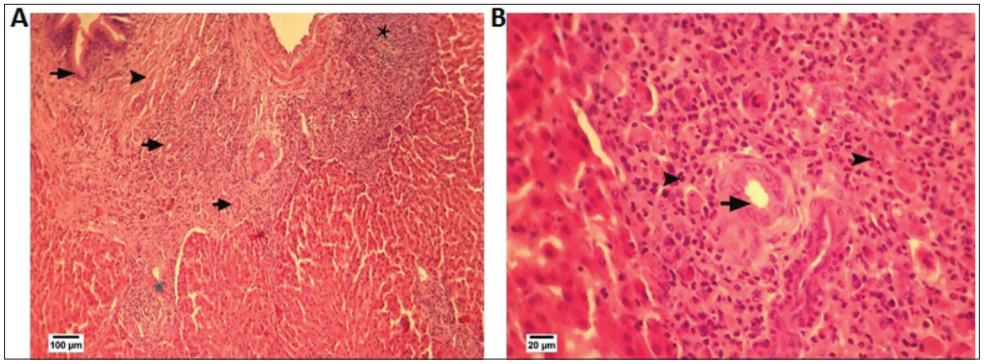
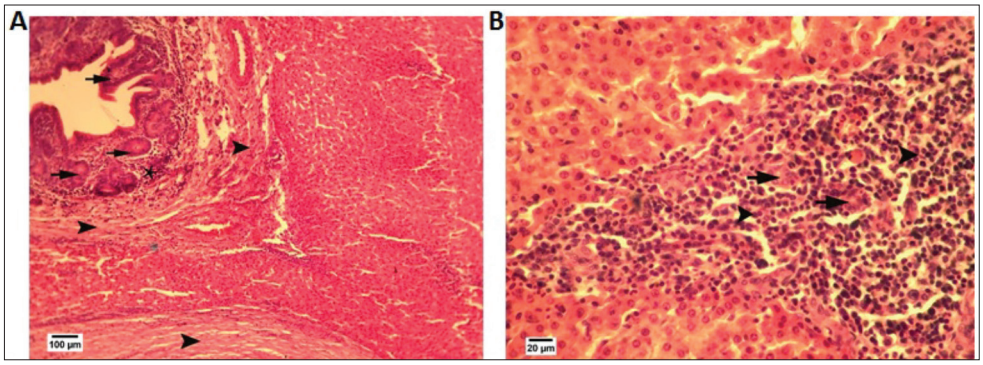


Fig 3. Liver tissue samples of sheep in the group 3 (Silymarin treatment: 280 mg/day) show pathological changes. *Arrowheads* point to the sites of fibrosis, hyperplasia, and inflammation (A, 100X & B, 400X)

Fig 4. Liver tissue samples of sheep in the group 4 (clorsulon treatment: 7.5 mg/kg/day) show pathological changes. *Arrows* point to the sites of fibrosis and hyperplasia, and *arrowheads* show inflammation (A, 100X &B, 400X)



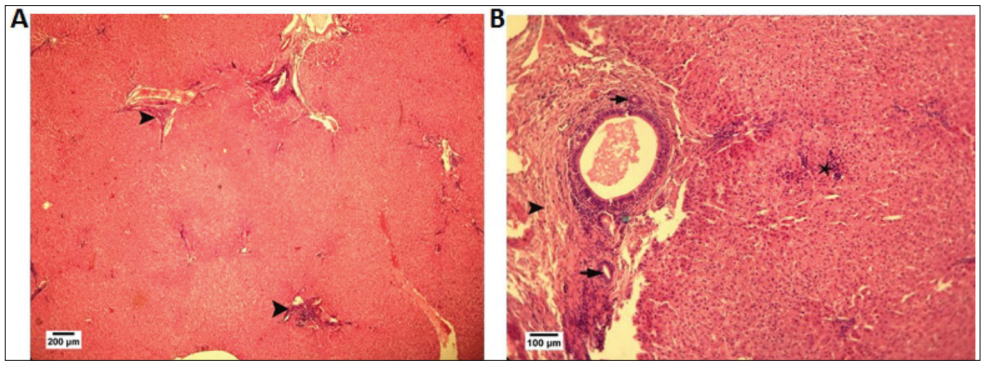


Fig 5. H&E stained liver tissue samples of sheep in the group 5 (clorsulon + silymarin treatment) show pathological changes. *Arrowheads* point to the sites of fibrosis, hyperplasia, and inflammation; (A, 40X & B, 100X)

the liver of the infected sheep *(Table 2*, P<0.0001).

The therapeutic effects of silymarin and clorsulon on the liver of *F. hepatica*-infected sheep showed in *Fig. 3* and *Fig. 4*, respectively. Although the bile duct hyperplasia reduced slightly in clorsulon-treated sheep *(Table 2)*, there were no significant changes in histopathological parameters following treatment with silymarin (group 3) or clorsulon alone (group 4) compared with the control group. The high rates of necrosis, fibrosis, hepatitis, and immune cell infiltration were still significant in these groups even after 21 days of treatment *(Table 2)*. The results indicated the hepatoprotective activity of silymarin when used in combination with clorsulon. In group 5 (clorsulon+silymarin treatment), the histopathological changes were considerable, as *Fig. 5* shows mild fibrosis, mild hepatitis *(Fig. 5A)*, mild bile duct hyperplasia, and less immune cell infiltration *(Fig. 5B)* after 21 days of combination treatment. *Table 2* shows the histopathological findings of the liver in all groups. As can be seen, all the pathological parameters were significantly lower in sheep treated with the combination of silymarin and clorsulon than in other infected groups after 21 days (P<0.0001).

DISCUSSION

Many studies have been investigated to find a suitable drug to reduce the hepatotoxicity effects of antiparasitic drugs in parasitic diseases such as fasciolosis. *F. hepatica* migration to the liver causes damage to the hepatic cell wall and leads to hepatic tissue necrosis and Biliary obstruction [20]. These effects cause changes in hepatic biochemical serum parameters. It showed that some hepatic biochemical parameters such as AST, ALT, and GGT are the reliable indicators of fascioliasis in sheep and could be used to determine the effectiveness of anthelmintic therapy in infected animals [1,16,21,22]. It also indicated that partial recovery of the liver following some treatments is associated with lower activities of these enzymes in

F. hepatica-infected sheep [21]. Although chemotherapy eliminates *F. hepatica* parasites significantly, less effective drugs may relieve the hepatic injuries and fibrosis due to parasites migration. Therefore, treatment targeting hepatic injuries caused by *F. hepatica* needs more investigations. Determination of plasma liver enzyme levels is a valuable method for assessing the efficacy of treatment in sheep infected with *F. hepatica* [1]. Lower serum levels of ALT, glutamic-oxaloacetic transaminase (GOT), and GGT are considered normal in the blood of sheep, while the higher serum activities of ALT, ALP, AST, and GGT in the infected animals are mainly the consequence of hepatic lesion and cholestasis [1].

In the current study, we evaluated the hepatoprotective activity of silymarin alone and in combination with clorsulon against *F. hepatica* in naturally infected sheep at different intervals for the first time. The observed elevation of serum transaminases (AST and ALT) and GGT in the infected animals before treatment could be related to the degenerative changes and hepatocellular necrosis produced by the migration of flukes through the liver parenchyma [23]. It has also been reported that the increased level of AST and GGT could be an indicator of chronic fascioliasis and liver cell damage [21,24,25] while using a hepatoprotective agent like silymarin could reduce the serum levels of AST and ALT [26]. Besides, the GGT level reduction after treatment could be the consequence of partial recovery of the liver [21].

In the present study, the serum levels of GGT, AST, and ALT decreased significantly following treatment with a combination of silymarin and clorsulon at the end of the treatment course (day 21). The reduction of these enzymes between the days 14 and 21 *(Table 1)* could be attributed to eliminating the parasite and preventing liver damage through maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes. In the present study, the histopathological findings also confirmed the biochemical results in which the liver damage improved after combination treatment *(Fig. 5)*. Another liver enzyme, ALP, also had a higher level in the infected groups than in healthy animals at the beginning of the study. The role of ALP is to transport metabolites across cell membranes. One of the most common reasons for hepatic disease is the pathological elevation of ALP levels [27]. Although the combination treatment with silymarin and clorsulon resulted in a significant reduction of this enzyme *(Table 1)*, it did not reach the baseline level at the end of the study, confirming that its level usually decreases slowly after resolution and thus, it needs more treatment time [27]. Another reason for the increase in this enzyme in group 4 is due to the hepatotoxicity effect of sulphonamide drugs [28] and the sulphonamide structure of clorsulon [3], which was reduced by the administration of silymarin and removal of the parasite in group 5.

Moreover, the decreases in serum total protein and albumin in animals infected with *F. hepatica* are observed previously [29]. Hypoalbuminemia causes by liver damage in acute and chronic fascioliasis and can cause biliary obstruction, cholangitis, anemia, destruction of liver tissue, and fibrosis [30]. Clorsulon and its combination with silymarin were effective to some extent, even at the beginning of the treatment. The increased levels of total protein and albumin at the end of treatment (day 21) by silymarin with/without clorsulon could be related to the hepatoprotective and anthelmintic activities of silymarin and clorsulon, respectively. The considerable increase in the total protein and albumin levels occurred at the end of the treatment by the combination therapy suggesting improved liver damage and less bile duct hyperplasia and fibrosis *(Fig. 5)*.

Furthermore, the biochemical analysis showed that total bilirubin was significantly higher ($P<0.05$) in the infected sheep than in the healthy group. This elevation may be attributed to the increased production of bilirubin because of hemolytic toxins produced by *F. hepatica* in the liver [23]. Since the combination treatment could affect the parasites' death and liver parenchyma at the same time, the lower levels of total bilirubin detected on day 21 ($P<0.05$). The improvement initiated on day seven and reached the average level similar to that of the healthy group on day 21 *(Table 1)*. All the results of the biochemical analysis were consistent with pathological findings *(Table 2)*.

The pathological results showed no significant changes following treatment with silymarin or clorsulon alone *(Fig. 1, 2, 3, 4)*, while the decrease in immune cells infiltration, bile duct hyperplasia, and fibrosis were considerable following the combination therapy *(Fig. 5)*. Silymarin and clorsulon alone were effective to some extent and reduced some of the biochemical parameters tested, but their combination was superior to each agent alone in terms of the histopathology of the damaged liver. Administration of silymarin, in addition to clorsulon, showed complete elimination of parasites. No visible eggs (data not shown) and healing of hepatic lesion injuries demonstrated that the use of silymarin along with clorsulon did not affect or interfere with the anthelmintic activity of clorsulon. The histopathological results of the present study confirmed biochemical findings of the current research, and measuring the studied parameters was useful to evaluate the effectiveness of hepatoprotective and anthelmintic agents.

The study, to the best of authors' knowledge, is the first report showing the hepatoprotective and anthelmintic efficacy of a combination of clorsulon and silymarin against *F. hepatica* in naturally infected sheep (Sangsari breed). Silymarin showed significant hepatoprotective activity when used in combination with clorsulon rather than alone administration. The study results also confirmed that serum levels of AST, ALT, ALP, GGT, albumin, protein, and bilirubin (total) are reliable indicators of sheep fasciolosis

and could be used to test the hepatoprotective effect of anthelmintic and hepatoprotective agents.

Acknowledgments and Funding

The current work was kindly supported by the Science and Research Branch, Islamic Azad University, Tehran, Iran. This study did not obtain any specific fund in the public, commercial, or not-for-profit sectors.

Conflict of Interest

All authors declare that there is no competing interest in the process of performing this manuscript.

Ethics Approval

The ethical committee approved the current work of the Science and Research Branch, Islamic Azad University. The number of approval: IR. IAU. SRB. REC. 1397. 12.

REFERENCES

1. Hodžić A, Zuko A, Avdić R, Alić A, Omeragić J, Jažić A: Influence of Fasciola hepatica on serum biochemical parameters and vascular and biliary system of sheep liver. *Iran J Parasitol,* 8 (1): 92-98, 2013.

2. Puspitasari S, Farajallah A, Sulistiawati E, Muladno: Effectiveness of ivermectin and albendazole against *Haemonchus contortus* in sheep in West Java, Indonesia. *Trop Life Sci Res,* 27 (1): 135-144, 2016.

3. Richter D, Richter J, Grüner B, Kranz K, Franz J, Kern P: In vitro efficacy of triclabendazole and clorsulon against the larval stage of *Echinococcus multilocularis. Parasitol Res,* 112 (4): 1655-1660, 2013. DOI: 10.1007/s00436-013-3321-7

4. Novobilský A, Solis NA, Skarin M, Höglund J: Assessment of flukicide efficacy against *Fasciola hepatica* in sheep in Sweden in the absence of a standardised test. *Int J Parasitol Drugs Drug Resist,* 6 (3): 141-147, 2016. DOI: 10.1016/j.ijpddr.2016.06.004

5. Karademir Ü, Gökbulut C, Boyacioglu M: The effect of fasting on the plasma disposition of albendazole in goats. *Kafkas Univ Vet Fak Derg,* 21 (6): 871-876, 2015. DOI: 10.9775/kvfd.2015.13789

6. Hanna REB, McMahon C, Ellison S, Edgar HW, Kajugu PE, Gordon A, Irwin D, Barley JP, Malone FE, Brennan GP, Fairweather I: *Fasciola hepatica:* A comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland sheep farms in Northern Ireland using faecal egg counting, coproantigen ELISA testing and fluke histology. *Vet Parasitol,* 207 (1-2): 34-43, 2015. DOI: 10.1016/j.vetpar.2014.11.016

7. Muhammad A, Ahmed H, Ali S, Saqlain M, Qayyum M, Simesk S: Monitoring of some anthelmintics against gastrointestinal nematodes in sheep and implications of resistance in Barani region, Pakistan. *Kafkas Univ Vet Fak Derg,* 25 (3): 373-378, 2019. DOI: 10.9775/kvfd.2018.21009

8. Dewick PM: Medicinal Natural Products: A Biosynthetic Approach. John Wiley & Sons, 2002.

9. Saller R, Melzer J, Reichling J, Brignoli R, Meier R: An updated systematic review of the pharmacology of silymarin. *Forsch Komplementmed,* 14 (2): 70-80, 2007. DOI: 10.1159/000100581

10. Avci H, Sekkin S, Boyacioglu M, Akşit H, Tunca R, Epikmen ET, Birincioğlu SS: Protective and antigenotoxic effects of silymarin and curcumin in experimental cyclophosphamide intoxication in rats. *Kafkas Univ Vet Fak Derg,* 22 (5): 693-701, 2016. DOI: 10.9775/kvfd.2016.15145

11. Kren V, Walterova D: Silybin and silymarin-New effects and applications. *Biomed Papers,* 149 (1): 29-41, 2005.

12. Vahabzadeh M, Amiri N, Karimi G: Effects of silymarin on metabolic syndrome: A review. *J Sci Food Agric,* 98 (13): 4816-4823, 2018. DOI: 10.1002/jsfa.9115

13. Erisir Z, Iflazoğlu Mutlu S, Şimsek ÜG, Çiftçi M, Azman MA, Benzer F, Erişir M: Effects of milk thistle *(Silybum marianum)* seed supplementation to high-calorie basal diets of quails on egg production, egg quality traits, hatchability and oxidative stress parameters. *Kafkas Univ Vet Fak Derg,* 22 (4): 577-584, 2016. DOI: 10.9775/kvfd.2016.15105

14. Mashayekhi M: Renoprotective effect of silymarin on gentamicin-induced nephropathy. *Afr J Pharm Pharmacol,* 6 (29): 2241-2246, 2012.

15. Martínez-Valladares M, Cordero-Pérez C, Rojo-Vázquez FA: Efficacy of an anthelmintic combination in sheep infected with *Fasciola hepatica* resistant to albendazole and clorsulon. *Exp Parasitol,* 136, 59-62, 2014. DOI: 10.1016/j.exppara.2013.10.010

16. Tsotetsi AM, Njiro S, Katsande TC, Moyo G, Baloyi F, Mpofu J: Prevalence of gastrointestinal helminths and anthelmintic resistance on small-scale farms in Gauteng province, South Africa. *Trop Anim Health Prod,* 45 (3): 751-761, 2013. DOI: 10.1007/s11250-012-0285-z

17. Beesley NJ, Caminade C, Charlier J, Flynn RJ, Hodgkinson JE, Martinez-Moreno A, Martinez-Valladares M, Perez J, Rinaldi L, Williams DJL: Fasciola and fasciolosis in ruminants in Europe: Identifying research needs. *Transbound Emerg Dis,* 65, 199-216, 2018. DOI: 10.1111/tbed.12682

18. Kaymaz MB, Kandemir FM, Pamukcu E, Eröksüz Y, Özdemir N: Effects of aqueous artichoke *(Cynara scolymus)* leaf extract on hepatic damage generated by alpha-amanitine. *Kafkas Univ Vet Fak Derg,* 23 (1): 155-160, 2017. DOI: 10.9775/kvfd.2016.16094

19. Ale-Ebrahim M, Eidi A, Mortazavi P, Tavangar SM, Tehrani DM: Hepatoprotective and antifibrotic effects of sodium molybdate in a rat model of bile duct ligation. *J Trace Elem Med Biol*, 29, 242-248, 2015. DOI: 10.1016/j.jtemb.2014.07.002

20. Deger Y, Ertekin A, Deger S, Mert H: Lipid peroxidation and antioxidant potential of sheep liver infected naturally with distomatosis. *Turkiye Parasitol Derg,* 32 (1): 23-26, 2008.

21. Kozat S, Denizhan V: Glucose, lipid, and lipoprotein levels in sheep naturally infected with *Fasciola hepatica. J Parasitol,* 96 (3): 657-659, 2010. DOI: 10.1645/GE-2104.1

22. Hodzic A, Zuko A, Avdic R, Alic A, Omeragic J, Jazic A: Influence of *Fasciola hepatica* on serum biochemical parameters and vascular and biliary system of sheep liver. *Iran J Parasitol,* 8 (1): 92-98, 2013.

23. Boone L, Meyer D, Cusick P, Ennulat D, Bolliger AP, Everds N, Meador V, Elliott G, Honor D, Bounous D, Jordan H: Selection and interpretation of clinical pathology indicators of hepatic injury in preclinical studies. *Vet Clin Pathol,* 34 (3): 182-188, 2005.

24. Mert H, Kozat S, Ekin S, Mert N, Yoruk I: Serum sialic acid, lipid-bound sialic acid levels in sheep naturally chronic infected with *Fasciola hepatica. J Health Sci Yuzuncu Yil Univ,* 9 (2): 40-46, 2006.

25. Denizhan V, Kozat S, Yilmaz AB: Investigation of serum amyloid A, haptoglobin and fibrinogen levels in sheep infected with *Fasciola hepatica. J Livest Sci,* 10, 53-58, 2019. DOI: 10.33259/JLivestSci.2019.53-58

26. de Avelar CR, Pereira EM, de Farias Costa PR, de Jesus RP, de Oliveira LPM: Effect of silymarin on biochemical indicators in patients with liver disease: Systematic review with meta-analysis. *World J Gastroenterol,* 23 (27): 5004-5017, 2017. DOI: 10.3748/wjg.v23.i27.5004

27. Giannini EG, Testa R, Savarino V: Liver enzyme alteration: A guide for clinicians. *CMAJ,* 172 (3): 367-379, 2005. DOI: 10.1503/cmaj.1040752

28. Singh A, Bhat TK, Sharma OP: Clinical biochemistry of hepatotoxicity. *J Clinic Toxicol,* S4:001, 2011. DOI: 10.4172/2161-0495.S4-001

29. Vengust G, Klinkon M, Bidovec A, Vengust A: *Fasciola hepatica:* Effects on blood constituents and liver minerals in fallow deer *(Dama dama). Vet Parasitol,* 112 (1-2): 51-61, 2003. DOI: 10.1016/S0304-4017(02)00413-2

30. Machicado C, Machicado JD, Maco V, Terashima A, Marcos LA: Association of *Fasciola hepatica* infection with liver fibrosis, cirrhosis, and cancer: A systematic review. *PLoS Negl Trop Dis,* 10 (9): e0004962, 2016. DOI: 10.1371/journal.pntd.0004962

Kafkas Univ Vet Fak Derg
26 (2): 287-294, 2020
DOI: 10.9775/kvfd.2019.22996

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Investigation of the Toxic Effects of Rhododendron Honey on Mouse Cardiac Muscle Tissue Lipids at Molecular Level [1] [2]

Gulgun CAKMAK-ARSLAN [1,a] Selin EMIR [1,b] Pinar GOC-RASGELE [2,c] Meral KEKECOGLU [1,d]

[1] *This work was supported by the Duzce University-Research Fund, BAP (2016.05.01.503)*
[2] *A part of this study was presented at the 3rd International Congress on Applied Biological Sciences in Afyon/Turkey on 09-12 July 2017*

[1] Department of Biology, Faculty of Arts and Sciences, Duzce University, TR-81620 Duzce - TURKEY
[2] Department of Biosystems Engineering, Faculty of Agriculture and Natural Sciences, Duzce University, TR-81620 Duzce - TURKEY
[a] ORCID: 0000-0003-4326-1780; [b] ORCID: 0000-0002-5819-6734; [c] ORCID: 0000-0002-7558-3138; [d] ORCID: 0000-0002-2564-8343

Article ID: KVFD-2019-22996 Received: 11.07.2019 Accepted: 26.11.2019 Published Online: 27.11.2019

How to Cite This Article

Cakmak-Arslan G, Emir S, Goc-Rasgele P, Kekecoglu M: Investigation of the toxic effects of rhododendron honey on mouse cardiac muscle tissue lipids at molecular level. *Kafkas Univ Vet Fak Derg,* 26 (2): 287-294, 2020. DOI: 10.9775/kvfd.2019.22996

Abstract

The purpose of this study is to investigate the effects of different concentrations of Rhododendron honey (RH) on mouse cardiac muscle lipids by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy at molecular level. For this purpose, a total of eighteen male *Mus musculus* mice were divided into three groups of six animals each, one being the control group and the others being the 25 and 50 mg/kg of RH-treated groups. RH was given via gavage and the cardiac muscles of these mice were investigated 24 h after the administration. The results revealed that 25 mg/kg of RH did not cause any significant effect except lipid peroxidation. However, 50 mg/kg RH caused increases in the amounts of saturated and unsaturated lipids, in the ratios of lipid/protein and CH_2/CH_3 and a decrease in the CH_3/lipid which all indicate a change in the lipid metabolism of the tissue. Moreover, the treatment with 50 mg/kg of RH caused lipid peroxidation, a decrease in lipid order and an increase in membrane dynamic. These results revealed that RH causes significant toxic effects on cardiac muscle tissue lipids and these effects are dose-dependent.

Keywords: *Rhododendron honey, Mad honey, ATR-FTIR spectroscopy, Heart, Cardiac muscle, Lipid*

Ormangülü Balı'nın Fare Kalp Kas Dokusu Lipitleri Üzerindeki Toksik Etkilerinin Moleküler Düzeyde İncelenmesi

Öz

Bu çalışmanın amacı, farklı konsantrasyonlardaki ormangülü balının (OB) fare kalp kası lipitleri üzerindeki etkilerinin Azaltılmış Toplam Yansıma-Fourier Dönüşüm Kızılötesi (ATR-FTIR) spektroskopisi ile moleküler düzeyde incelenmesidir. Bu amaç doğrultusunda, toplam on sekiz adet *Mus musculus* erkek fare, her biri altı hayvan içeren, birisi kontrol ve diğerleri 25 ve 50 mg/kg OB uygulanmış gruplar olmak üzere üç gruba ayrıldı. OB gavaj yoluyla verildi ve bu farelerin kalp kasları uygulamadan 24 saat sonra incelendi. Sonuçlar 25 mg/kg OB'nin kalp kası lipitleri üzerinde lipit peroksidasyonu dışında herhangi anlamlı bir değişikliğe sebep olmadığını ortaya çıkarmıştır. Ancak 50 mg/kg OB doymuş ve doymamış lipitlerin miktarlarında, lipit/protein oranında ve CH_2/CH_3 oranında artışa ve CH_3/lipit oranında azalmaya sebep olmuştur. Tüm bunlar dokunun lipit metabolizmasında bir değişiklik olduğunu göstermektedir. Ayrıca 50 mg/kg OB lipit peroksidasyonuna, lipit düzeninde bir azalmaya ve membran düzeninde bir artışa sebep olmuştur. Bu sonuçlar, OB'nin kalp kası lipitleri üzerinde önemli toksik etkiler meydana getirdiğini ve bu etkilerin doza bağlı olduğunu ortaya çıkarmıştır.

Anahtar sözcükler: *Ormangülü balı, Deli bal, ATR-FTIR spektroskopisi, Kalp, Kalp kası, Lipit*

INTRODUCTION

Rhododendron honey (RH), also known as mad honey, is obtained from plants belonging to the genus Rhododendron in the Ericaceae family. The rhododendrons growing extensively in the Black Sea region of Turkey show also distribution in China, Tibet, Nepal, Tropical Asia, Europe and North America [1]. RH causes poisoning in humans and animals due to the toxic compound grayanotoxin (GTX) found in some species of the Rhodendron genus. However,

İletişim (Correspondence)
+90 380 5412404/3991 Fax: +90 380 5412403
gulguncakmak@duzce.edu.tr

in different regions of the world, this honey is widely used in the treatment of various disorders such as hypertension, gastrointestinal complaints, sexual dysfunction toothache, colds, etc.[2].

The effect of GTX is predominantly on the voltage-dependent sodium channel. It binds to the sodium channel in the open position and the sodium channel becomes incapable of closing. As a result, action potential enters a prolonged period of hyperpolarization process [3,4]. Through this action mechanism, GTX affects the heart directly and causes a wide range of systemic effects including hypotension, arrhythmia, nausea and a reduction in spontaneous motion by affecting the central nervous system [1]. Most of the published studies on RH have presented case reports either on patient complaints after eating the honey or cases of patients treated at emergency services [3]. In clinical studies examining the effects of RH on the cardiac system, it has been reported that hypotension and bradycardia were seen in more than 90% of the patients [5].

In the literature, experimental studies on the effects of RH on the cardiac system are extremely limited in number, compared to the clinical trials on the subject. For example, Onat et al.[6] observed obesity-related hypotension, bradycardia and respiratory rate depression in RH-treated mice. In another study, blood pressure and heart rate were measured in rats with experimental hypertension and it was reported that RH reduced the blood pressure and heart rate in the hypertensive rats [7]. Zushi et al.[8] have reported that the effect of GTX on the neuromuscular junction was achieved by increased permeability of the membrane to sodium. In a recent experimental study investigating the dose-related cardiovascular effects of GTX-III, it was reported that it could be lethal at high doses due to cardiac arrest [5].

Fourier transform infrared (FTIR) spectroscopy is a high-tech tool that gathers valuable information about biological tissues and membranes by measuring the vibrations of molecules [9]. By using the attenuated total reflection (ATR) unit in FTIR spectroscopy, it is possible to detect spectral changes in biological tissues faster and more accurately, regardless of sample thickness [10]. The ATR-FTIR spectroscopy is a unique technique that enables the detection of absorption bands of lipids, proteins, carbohydrates and nucleic acids in biological systems in a single spectrum and at the same time, monitoring these molecules without using any labeling technique [10]. The main advantage of this technique is that the samples can be investigated without any preparation processes by placing them directly on the ATR crystals.

Recent studies have revealed the importance of intracellular myocardial and pericardial lipid deposits, showing that even the smallest changes in these fat deposits cause significant changes in cardiac performance [11]. It is known that RH leads to cardiac side effects and functional disorders; however, there is no study reporting the effects of RH on the structure and function of lipids in the cardiac muscle. The ability of a tissue to function properly is related to its structure and ATR-FTIR spectroscopy gives information about the structure of the tissue at molecular level. In this study, we aimed to reveal the molecular effects of two different doses of RH on the composition, structure and function of the cardiac muscle lipids by using ATR-FTIR spectroscopy. To the best of our knowledge, this is the first study to investigate the effects of RH on cardiac muscle lipids at the molecular level.

MATERIAL and METHODS

The RH that we used in our study was obtained from Düzce beekeepers. As a result of the palynological analysis, the honey sample was confirmed to dominantly (≥45%) consist of the *Rhododendron ponticum* pollen [12].

The amount of GTX-I and GTX-III in the RH used in this study was detected by liquid chromatography-mass/mass spectrometry (LC-MS/MS) using the method developed by Kaplan et al.[13]. The limit of detection and the limit of quantification values of the validated analysis method were 0.0033 mg/kg and 0.01 mg/kg, respectively. The GTX-I and GTX-III amounts of the samples were found to be 32 and 8 µg/g, respectively, which were higher than the average values reported previously [13] and in parallel with the amounts of toxic substances in RH used in other studies [14-16].

All experimental procedures were approved by the Abant İzzet Baysal University Medical Faculty Experimental Animals Ethics Committee (2015/42). 18 male *Mus musculus* mice (20-25 g), 8-12 weeks old, were used. The animals were housed in a 12 h light - 12 h dark cycle at a constant room temperature (22±2°C) and fed with mouse food and water. The mice were divided into three groups: the control group (n=6), the 25 mg/kg RH-treated group (n=6) and the 50 mg/kg RH-treated group (n=6). The different concentrations of RH were prepared by dissolving in water and they were administered via gavage to the animals in 0.01 mL per gram body weight ratio. 24 h after administration, the animals were decapitated and their heart tissues were removed and stored at -80°C for latter ATR-FTIR spectroscopy experimentation.

The mouse cardiac muscle spectra were collected with a Spectrum Two FTIR spectrometer equipped with an ATR accessory (Perkin-Elmer Ltd., UK). 0.5 × 0.5 × 0.1 cm sized sections were cut from the myocardial layer of the heart for the ATR-FTIR studies and placed directly on the diamond/zinc-selenide crystals of the ATR unit. For each sample, spectra were obtained at 4 cm^{-1} resolution and 100 force gauge with 64 scans between the 4000-900 cm^{-1} wavenumbers. The analyses of spectral bands were performed using Perkin Elmer software programme. Bandwidth and band wavenumber values were calculated at 75% of the band height [9,10].

For quantitative comparison between control and treated samples, the areas under the lipid bands and the area ratios of some specific infrared bands were calculated. To examine the level of the lipid peroxidation of the system, the area under the olefinic=CH band and the unsaturated/saturated lipid ratio (the area of the olefinic=CH stretching band/the sum of the areas of the saturated lipid bands), which are the parameters used as an index for the determination of lipid peroxidation in FTIR studies, were used [9,17]. To find out the changes in the amount of saturated lipids, the areas under the CH_3 antisymmetric (antisym) stretching, CH_2 antisym stretching and CH_2 symmetric (sym) stretching bands and the ratio of CH_2 sym/CH_2 sym + CH_2 antisym stretching vibrations were evaluated [9,18,19]. To have information about the amount of triglycerides and cholesterol in the system, the area under the C=O ester stretching band was analyzed [20]. To determine the changes in the chain length of the membrane phospholipids, the CH_2/CH_3 ratio (the area of the CH_2 antisym stretching/the area of the CH_3 antisym stretching) [21] and in the methyl concentration, CH_3/lipid (the area of the CH_3 antisym stretching band/the sum of the areas of saturated lipid bands) ratio [20] were calculated. To compare the relative changes in lipid and protein concentrations in the system, the area ratios of the sum of the saturated lipid bands (CH_3 antisym, CH_2 antisym and CH_2 sym stretching bands) and proteins (Amide II band) were obtained [21]. To have information about membrane order and membrane fluidity, the wavenumber and bandwidth of the CH_2 antisym stretching band were analyzed, respectively [9].

Power analysis was performed to estimate the test power considering the sample size of each experimental group (n=6) for a power of 80% to achieve significant statistical differences at the 5% significance level and the calculated effect size value was 1.55. Mann-Whitney U test, which is a non-parametric test used to compare two independent groups that do not require large normally distributed samples, was performed to test the significance of the differences between the control and RH-treated groups two by two; that is, between the control and 25 mg/kg RH-treated groups and the control and 50 mg/kg RH-treated groups. The P values less than 0.05 were considered statistically significant.

RESULTS

Fig. 1 shows the ATR-FTIR spectrum of a control mouse cardiac muscle in the wavenumber range of 4000-900 cm^{-1}. The main bands on the figure are numbered and their definitions are given in *Table 1* according to the literature.

The cardiac muscle spectrum shown in *Fig. 1* can be examined in three different regions: The large band located at 3700-3030 cm^{-1} (band No. 1) mainly receives signals from the N-H groups of proteins with the little contribution from O-H stretching of polysaccharides, carbohydrates and water [20]. The 3025-2800 cm^{-1} region (band No's. 2-6) is called the C-H stretching region and generally provides information about lipids [18]. The 1800-800 cm^{-1} region (band No's. 7-17), which is called the fingerprint region, receives signals mostly from proteins and nucleic acids and in small quantities from lipids and carbohydrates [22]. In this study, since we aimed to collect information about the effects of different concentrations of RH on cardiac muscle lipids, the detailed analyses were performed mainly in the region of 3025-2800 cm^{-1}. *Fig. 2* shows the average ATR-FTIR spectra in the 3025-2800 cm^{-1} region of the cardiac muscle of control and 25 and 50 mg/kg RH-treated mice.

To obtain information about the changes in the concentrations of lipid molecules after RH treatment, analyses of the areas under the lipid bands in the FTIR spectra were performed [18]. The changes in the band area values of the major functional groups are given in *Table 2*. As seen in *Table 2*, the area under the olefinic=CH stretching band (band No. 2) increased significantly in the 50 mg/kg RH-treated group indicating an increase in the amount of unsaturated lipids. As also seen in *Fig. 2* and *Table 2*, the region under the saturated lipid bands (band No's. 3, 4, 6) increased significantly in the 50 mg/kg RH group. This result suggested that the 50 mg/kg RH administration caused an increase in the amount of saturated lipid in the cardiac muscle. The result derived from the analysis of the lipid bands in the C-H region was supported by the

Fig 1. ATR-FTIR spectrum of control mouse cardiac muscle in the 4000-900 cm^{-1} wavenumber region

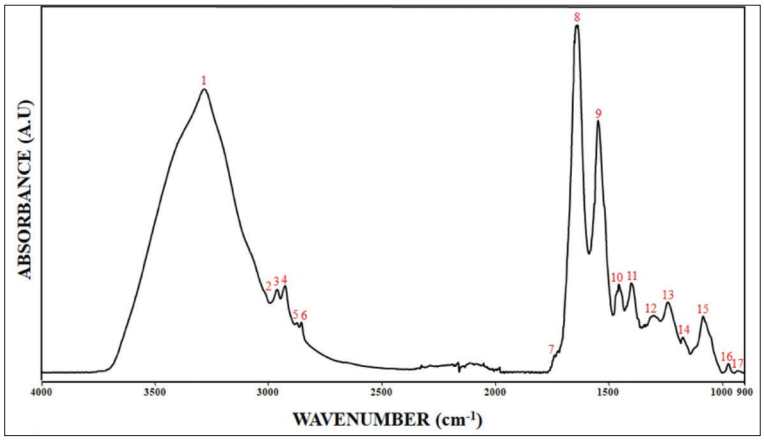


Table 1. *General band assignment of ATR-FTIR spectrum of cardiac muscle tissue based on literature* [18,20,22]

Band No	Wavenumber (cm^{-1})	Definition of the Assignment
1	3283	Amide A: Mainly N-H stretching of hydrogen-bonded amide groups of proteins with the little contribution from O-H stretching of polysaccharides, carbohdrates and water
2	3011	Olefinic=CH stretching: Unsaturated lipids
3	2959	CH_3 antisymmetric stretching: Mainly lipids with little contribution from proteins, carbohydrates and nucleic acids
4	2924	CH_2 antisymmetric stretching: Mainly lipids with little contribution from proteins, carbohydrates and nucleic acids
5	2874	CH_3 symmetric stretching: Mainly proteins with little contribution from lipids, carbohydrates and nucleic acids
6	2855	CH_2 symmetric stretching: Mainly lipids with little contribution from proteins, carbohydrates and nucleic acids
7	1738	C=O (carbonyl) ester stretching: Ester functional groups in phospholipids, triglycerides and cholesterol esters
8	1641	Amide I: C=O stretching in proteins (80%)
9	1545	Amide II: Proteins (60% N-H bending, 40% C-N stretching)
10	1454	CH_2 bending: Mainly lipids with little contribution from proteins
11	1396	COO^- symmetric stretching: Fatty acids and amino acid side groups
12	1302	Amide III: Proteins (40%C-N stretching, 30% N-H bending, 20% C-C stretching)
13	1238	PO^-_2 antisymmetric stretching: Mainly nucleic acids with some contribution from phospholipids
14	1172	CO-O-C antisymmetric stretching: Phospholipids, cholesteryl ester and nucleic acids
15	1080	PO^-_2 symmetric stretching: Nucleic acids and phospholipids; C-O stretching: glycogen, polysaccarides and glycolipids
16	972	C-N^+-C stretching: Nucleic acids, ribose-phosphate main chain vibrations of RNA
17	931	z-type DNA

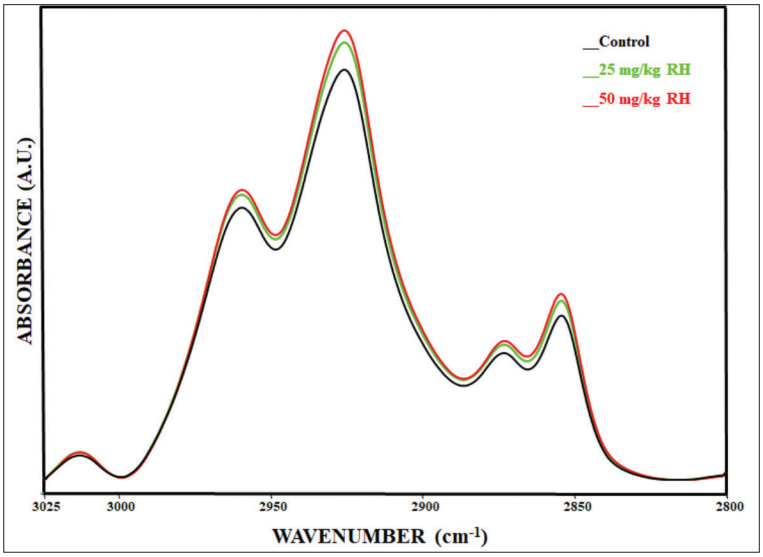


Fig 2. Average infrared spectra in the 3025-2800 cm^{-1} spectral region of cardiac muscle of control, 25 mg/kg and 50 mg/kg RH-treated mice (The spectra were normalized with regard to the amide I band)

increase in the value of the C=O ester stretching band located at 1738 cm^{-1} (band No. 7). As shown in *Table 2*, a significant increase was observed in the area value of this band in the 50 mg/kg RH-treated group.

In order to evaluate the effects of RH on the structure and composition of the cardiac muscle lipids, the area ratios of some specific lipid functional groups were also evaluated [21]. The band area ratios calculated for this purpose are given in *Table 3*. As shown in *Table 3*, the ratio of CH_2 sym/CH_2 sym + CH_2 antisym stretching vibrations increased significantly in the 50 mg/kg RH-treated group. This increase confirmed the increase in the amount of saturated lipids in the system obtained from the analysis of the lipid band areas. The unsaturated/saturated lipid ratio and the CH_2/CH_3 ratio increased significantly in 50 mg/kg RH-treated groups compared to the control group. A significant decrease in CH_3/lipid ratio suggested a decrease in the amount of methyl groups in the 50 mg/kg RH-treated group. The lipid/protein ratio increased significantly in the 50 mg/kg RH-treated group.

The changes in the wavenumber and bandwidth values of the CH_2 antisym stretching band are given in *Table 3*. As seen from this table, the wavenumbers of the CH_2 antisym stretching band shifted towards higher values in the 50

Table 2. *Changes in the band area values of the major lipid bands of the ATR-FTIR spectra of the cardiac muscle of control, 25 mg/kg and 50 mg/kg RH-treated mice*

Band No	Functional Group	Wavenumber (cm^{-1})	Control	25 mg/kg RH	P Value[a]	50 mg/kg RH	P Value[b]
2	Olefinic=CH Stretch.	3011	0.021±0.004	0.030±0.006	0.100	0.037±0.005	0.034*
3	CH_3 Antisym. Stretch.	2959	0.433±0.018	0.448±0.038	0.807	0.461±0.018	0.034*
4	CH_2 Antisym. Stretch.	2924	0.815±0.025	0.855±0.072	0.376	0.896±0.063	0.043*
6	CH_2 Sym. Stretch.	2855	0.125±0.008	0.136±0.015	0.167	0.143±0.010	0.015*
7	Carbonyl Ester Stretch.	1738	0.623±0.012	0.655±0.037	0.165	0.666±0.036	0.006**

*Values are given as "mean ± standard deviation" for each group. Degree of significance was denoted as *P<0.05, **P<0.01.* ***P value[a]:*** *P values are from the comparison of the control and 25 mg/kg RH-treated mice;* ***P value[b]:*** *P values are from the comparison of the control and 50 mg/kg RH-treated mice*

Table 3. *Changes in the band area ratio, wavenumber and bandwidth values of various functional groups of the ATR-FTIR spectra of the cardiac muscle of control, 25 mg/kg and 50 mg/kg RH-treated mice*

Functional Group		Control	25 mg/kg RH	P Value[a]	50 mg/kg RH	P Value[b]
Band Area Ratio	CH_2 sym/CH_2 sym + CH_2 antisym	0.132±0.002	0.134±0.004	0.574	0.137±0.003	0.045*
	Unsaturated/Saturated lipid	0.019±0.0008	0.021±0.001	0.030*	0.022±0.001	0.024*
	CH_2/CH_3	1.876±0.030	1.904±0.05	0.298	1.939±0.040	0.008**
	CH_3/Lipid	0.312±0.003	0.310±0.006	0.688	0.308±0.002	0.013*
	Lipid/Protein	0.105±0.003	0.108±0.008	0.872	0.113±0.004	0.030*
Wavenumber	CH_2 antisym. str.	2927.613±0.198	2927.751±0.393	0.521	2927.851±0.076	0.045*
Bandwidth	CH_2 antisym. str.	11.355±0.142	11.380±0.136	0.872	11.525±0.094	0.045*

*Values are given as "mean ± standard deviation" for each group. Degree of significance was denoted as * P<0.05, ** P<0.01.* ***P value[a]:*** *P values are from the comparison of the control and 25 mg/kg RH-treated mice;* ***P value[b]:*** *P values are from the comparison of the control and 50 mg/kg RH-treated mice*

mg/kg RH group compared to the control group. As seen from the same table, the bandwidth of this band increased significantly in the 50 mg/kg RH-treated group.

DISCUSSION

It has been reported that consuming more than 50 mg/kg (approximately 1 teaspoon for humans) of RH may cause serious poisoning [23], with symptoms appearing within 20 min to 3 h and lasting for 1-2 days [24]. For this reason, in this study, doses of 50 mg/kg, which is accepted as the toxic dose limit, and 25 mg/kg, which is ½ of the toxic dose, were selected for administration to the animals. Doses similar to those used in this study have also been used in previous studies [7,25,26].

Since unsaturated fatty acids are highly sensitive to lipid peroxidation, the area under the olefinic=CH band and the unsaturated/saturated lipid ratio in FTIR spectroscopy studies are used as an index for the determination of lipid peroxidation [9,17]. In this study, significant increases were observed in the olefinic=CH band area in the 50 mg/kg RH-treated group and unsaturated/saturated lipid ratio in the 25 and 50 mg/kg RH-treated groups. This increase may have been due to an increase in lipid peroxidation end products in the cardiac tissue as a result of an elevated level of lipid peroxidation in the cardiac muscle of the 50 mg/kg RH-treated group [21]. It has been known that lipid peroxidation occurs in the double bonds in the acyl chains of polyunsaturated fatty acids [27] and it results in the loss of olefinic bonds [28]. However, according to the results of our experiments, instead of a decrease, an increase was observed in the olefinic groups. This result indicated that the amount of unsaturated fatty acid lost during lipid peroxidation reactions was compensated by the double bonds present in the lipid peroxidation end products, as reported in some previous FTIR spectroscopy studies [17,21]. In addition, in the present study, it was determined that the area under the C=O ester stretching band increased in the spectrum of the 50 mg/kg RH-treated group. Since there are abundant C=O (carbonyl) groups in lipid peroxidation end products, such as malondialdehyde (MDA), an increase in the area value of this band also indicates an increase in lipid peroxidation end products in the system [29].

It has been known that a large part of the heart energy requirement is provided by the oxidation of free fatty acids and fatty acids are extremely sensitive to the harmful effects of free radicals [11]. Under normal conditions, free radicals resulting from oxidative stress are produced in small amounts in all body cells. However, these radicals are inactivated by antioxidant enzymes and thus prevented from attacking other molecules in the cell [30]. If the production of free radicals increases rapidly for any reason, the mechanism of inactivation cannot compensate and these radicals react with other molecules in the cell and disrupt their structure. For example, the hydroxyl radical, one of the oxidative stress-producing free radicals, reacts with long chain fatty acids and causes lipid peroxidation [30]. As mentioned above, GTX increases the sodium permeability

of the membrane [4]. The increase in intracellular sodium concentration may affect the intracellular and extracellular sodium/calcium exchange mechanisms [31]. In this way, the intracellular calcium concentration increases, while the sodium level decreases on the other side. The increase in intracellular calcium concentration is known to be one of the main mechanisms triggering the formation of free radicals [32]. It is also known that exposure to GTX can lead to dysfunction in organs such as the liver and kidneys and functional defects in organs and impaired balance in the biological system may lead to free radical formation [16]. On the other hand, it is known that GTX plays an important role in cardiotoxicity by binding to the muscarinic M2 receptors [33]. The increased vagal tone of GTX leads to the condensation of the cholinergic effect, especially in the cardiovascular system and to the impairment of the physiological balance of other systems [33]. These may indirectly lead to the degradation of the oxidant/anti-oxidant balance at the cellular level, the weakening of the cellular antioxidant defense system and the formation of free radicals. As a result, the increase in the area values of olefinic=CH and carbonyl ester stretching bands and the ratio of unsaturated/saturated lipids observed in this study could be attributed to lipid peroxidation as a consequence of the attack of free radicals to the fatty acids in the system. Our results showed that the mechanism of action of GTX was related to the potential for oxidative stress formation. In a study conducted by Eraslan et al.[34], in accordance with our results, oxidative stress induced by different doses of RH caused increases in the oxidative stress markers such as MDA, NO (nitric oxide) and HNE (4-hydroxynonenal) along with changes in enzyme levels. Similarly, lipid peroxidation induced by GTX in various cells and tissues determined via increase in MDA level was reported by Silici et al.[35] In the same study, an increase in antioxidant enzyme levels in the plasma and in various tissues was also observed. Since antioxidant enzymes play an active role in converting harmful free radicals into less harmful or harmless compounds, this increase can be regarded as an indication of lipid peroxidation due to oxidative stress.

According to the results of the present study, the amount of saturated lipids increased significantly in the 50 mg/kg RH-treated group. This result was supported by the increase observed in the chain length, which is determined by the increase in the CH_2/CH_3 ratio, in 50 mg/kg RH-treated group [20]. The increase in lipid chain length may have been due to an increase in lipid content in the cardiac muscle tissue after 50 mg/kg RH administration. As systems with longer chained lipids contain relatively fewer methyl groups, the CH_3/lipid ratio decreased when the lipid chain length increased [21]. Thus, the observed reduction in the CH_3/lipid ratio following the administration of 50 mg/kg RH confirmed the conclusion that the lipid chain length had increased. These results suggested that the dose of 50 mg/kg RH caused changes in lipid metabolism in the cardiac muscle, resulting in the accumulation of lipids. The physiological balance between lipid uptake and oxidation prevents the accumulation of excess lipids. If this balance is impaired for any reason, accumulation of myocardial lipid is observed and this situation leads to various pathological responses [36]. Fatty acids significantly affect crucial membrane functions like membrane fluidity and membrane structure stability, membranous ion and substance transport and cardiac electrophysiology, which is essential for cardiac function and excitability. In addition, they play a role as regulatory molecules in the formation of oxidative and ischemic damage, as a secondary messenger in cell signaling and transduction and as an effector in apoptosis [37]. For this reason, abnormalities in fatty acid metabolism affect the structure and function of the cardiac system adversely. In a study conducted by Oztasan et al.[25], it has been demonstrated that RH caused changes in the lipid metabolism. In that study, diabetic rats were found to have reduced blood lipid levels after RH administration [25]. RH may cause a decrease in the lipid content in the blood due to this effect, which may in turn lead to an increase in the amount of lipids in tissues such as cardiac muscle. Most of these lipids that accumulate in the cardiac muscle tissue affect cardiac function in the worst way and cause myocardial structural damage, such as cardiac fibrosis, myocyte apoptosis and decreased contraction thought to be caused by frequent mitochondrial disorders [36]. Since consumption of RH in high amounts is known to cause cardiac side effects and functional disorders [38], the lipids accumulated in the tissue may contribute to all these functional disorders.

In the current study, the lipid/protein ratio in the 50 mg/kg RH-treated group was significantly increased. This finding pointed out a change in the lipid asymmetry in the cardiac cell membranes and supported the conclusion that there has been a change in lipid metabolism in the system after the 50 mg/kg RH administration. It is known that changes in lipid asymmetry cause significant alterations in intracellular and intercellular ion concentrations and ultimately in membrane function [39]. The wavenumber of the CH_2 antisym stretching band in the FTIR spectrum gives information about the order and disorder status of the lipids in the membrane. In the present study, the wavenumber of the CH_2 antisym stretching band of the 50 mg/kg RH-treated group showed a significant shift towards higher values. This finding suggested that the 50 mg/kg RH administration caused a reduction in the membrane order [9]. The reduction in membrane order may have been related to lipid peroxidation caused by oxidative stress induced by RH [40]. In addition, the bandwidth of the CH_2 antisym stretching band was significantly increased in the 50 mg/kg RH group. This increase in bandwidth indicated an increase in membrane fluidity [9]. The observed changes in lipid fluidity may be due to the changes in the lipid composition, lipid concentrations and their changes relative to each other and to a change in the lipid/protein ratio [9]. It is known that the effects of GTX on the

skeleton and cardiac muscle are all due to changes in the cell membrane [41]. As GTX is a fat-soluble toxin, the order and fluidity of the cell membrane is very important for permeability to GTX. As a result of the action mechanism of GTX, some changes may occur in the cytosolic calcium concentration which plays important roles in events such as muscle contraction, cell division, apoptosis, and neurotransmitter release [4,42]. In our study, the decrease in the membrane order and the increase in the membrane fluidity were factors that increased the membrane permeability. Therefore, our results are consistent with the findings that RH affects the cell membrane, resulting in changes in intracellular ion concentration. Our findings showed that the 50 mg/kg RH dose disrupted the normal functioning of the cell membrane due to significant changes in membrane order and fluidity. These adverse effects on the order and fluidity of the cell membrane could be one of the toxic mechanisms of RH on the cardiac muscle.

Our results revealed that administration of 25 mg/kg of RH did not cause any significant change in the mouse cardiac muscle lipids except for an increase in the unsaturated/saturated fatty acid ratio, which is an indication of lipid peroxidation. However, 50 mg/kg RH induced significant changes on the tissue lipids at molecular level together with lipid peroxidation. The results of our study demonstrating the toxic effects 50 mg/kg RH on the structure, composition and dynamics of cardiac muscle lipids are crucial to reveal the action mechanism of RH on the functions of the cardiac muscle and have been reported for the first time. The global changes in lipids and membranes observed in this study may reflect one of the main mechanisms of the toxicity of GTX in RH. In addition, the results of this study show that the amount of RH is important and the induced effects depend on the dose consumed.

REFERENCES

1. Ullah S, Khan SU, Saleh TA, Fahad S: Mad honey: Uses, intoxicating/poisoning effects, diagnosis, and treatment. *RSC Adv,* 8 (33): 18635-18646, 2018. DOI: 10.1039/C8RA01924J

2. Kukner A, Ilter G, Soyler G, Goc Rasgele P, Kekecoglu M, Kambur M: The Effect of rhododendron honey on mice liver tissue. *Int J Morphol,* 34 (3): 842-847, 2016. DOI: 10.4067/S0717-95022016000300004

3. Gunduz A, Turedi S, Russell RM, Ayaz FA: Clinical review of grayanotoxin /mad honey poisoning past and present. *Clin Toxicol,* 46 (5): 437-442, 2008. DOI: 10.1080/15563650701666306

4. Kim SE, Shin MC, Akaike N, Kim CJ: Presynaptic effects of grayanotoxin III on excitatory and inhibitory nerve terminals in rat ventromedial hypothalamic neurons. *Neurotoxicology,* 31 (2): 230-238, 2010. DOI: 10.1016/j.neuro.2009.12.006

5. Yılmaz İ, Kaya E, Yaykaşlı KO, Türker Y: Sıçanlarda intravenöz uygulanan deli bal toksini grayanotoksin-III'ün doza bağımlı kardiyak etkileri. *Behcet Uz Cocuk Hast Derg,* 7 (2): 121-128, 2017. DOI: 10.5222/buchd.2017.121

6. Onat FY, Yegen BC, Lawrence R, Oktay A, Oklay S: Mad honey poisoning in man and rat. *Rev Environ Health,* 9 (1): 3-10, 1991. DOI: 10.1515/reveh.1991.9.1.3

7. Oztasan N, Songur A: The use of "mad honey" as an antihypertensive agent in rats-a preliminary study. *Kocatepe Tip Derg,* 8 (1): 55-58, 2007.

8. Zushi S, Miyagawa J, Yamamoto M, Kataoka K, Seyama I: Effect of grayanotoxin on the frog neuromuscular junction. *J Pharmacol Exp Ther,* 226 (1): 269-275, 1983.

9. Cakmak G, Severcan M, Zorlu F, Severcan F: Structural and functional damages of whole body ionizing radiation on rat brain homogenate membranes and protective effect of amifostine. *Int J Radiat Biol,* 92 (12): 837-848, 2016. DOI: 10.1080/09553002.2016.1230237

10. Ozek NS, Bal IB, Sara Y, Onur R, Severcan F: Structural and functional characterization of simvastatin-induced myotoxicity in different skeletal muscles. *Biochim Biophys Acta,* 1840 (1): 406-415, 2014. DOI: 10.1016/j.bbagen.2013.09.010

11. Wolf P, Winhofer Y, Krssak M, Krebs M: Heart, lipids and hormones. *Endocr Connect,* 6 (4): R59-R69, 2017. DOI: 10.1530/EC-17-0031

12. Maurizio A, Hodges FED: Pollen analysis of honey. *Bee World,* 32 (1): 1-5, 1951. DOI: 10.1080/0005772x.1951.11094660

13. Kaplan M, Olgun EO, Karaoglu O: Determination of grayanotoxins in honey by liquid chromatography tandem mass spectrometry using dilute-and-shoot sample preparation approach. *J Agric Food Chem,* 62 (24): 5485-5491, 2014. DOI: 10.1021/jf501560t

14. Eraslan G, Kanbur M, Karabacak M, Arslan K, Silig Y, Soyer Sarica Z, Tekeli MY, Tas A: Effect on oxidative stress, hepatic chemical metabolizing parameters, and genotoxic damage of mad honey intake in rats. *Hum Exp Toxicol,* 37 (9): 991-1004, 2018. DOI: 10.1177/0960327117745691

15. Silici S, Yonar ME, Sahin H, Atayoglu TA, Ozkok D: Analysis of grayanatoxin in Rhododendron honey and effect on antioxidant parameters in rats. *J Ethnopharmacol,* 156, 155-161, 2014. DOI: 10.1016/j.jep.2014.08.027

16. Silici S, Dogan Z, Sahin H, Atayoglu T, Yakan B: Acute effects of grayanotoxin in rhododendron honey on kidney functions in rats. *Environ Sci Pollut Res,* 23 (4): 3300-3309, 2016. DOI: 10.1007/s11356-015-5534-z

17. Severcan F, Gorgulu G, Gorgulu ST, Guray T: Rapid monitoring of diabetes-induced lipid peroxidation by Fourier transform infrared spectroscopy: Evidence from rat liver microsomal membranes. *Anal Biochem,* 339 (1): 36-40, 2005. DOI: 10.1016/j.ab.2005.01.011

18. Elibol-Can B, Jakubowska-Dogru E, Severcan M, Severcan F: The effects of short-term chronic ethanol intoxication and ethanol withdrawal on the molecular composition of the rat hippocampus by FT-IR spectroscopy. *Alcohol Clin Exp Res,* 35 (11): 2050-2062, 2011. DOI: 10.1111/j.1530-0277.2011.01556.x

19. Gurbanov R, Bilgin M, Severcan F: Restoring effect of selenium on the molecular content, structure and fluidity of diabetic rat kidney brush border cell membrane. *Biochim Biophys Acta,* 1858 (4): 845-854, 2016. DOI: 10.1016/j.bbamem.2016.02.001

20. Cakmak G, Miller LM, Zorlu F, Severcan F: Amifostine, a radioprotectant agent, protects rat brain tissue lipids against ionizing radiation induced damage: An FTIR microspectroscopic imaging study. *Arch Biochem Biophys,* 520 (2): 67-73, 2012. DOI: 10.1016/j.abb.2012.02.012

21. Cakmak G, Zorlu F, Severcan M, Severcan F: Screening of protective effect of amifostine on radiation-induced structural and functional variations in rat liver microsomal membranes by FT-IR spectroscopy. *Anal Chem,* 83 (7): 2438-2444, 2011. DOI: 10.1021/ac102043p

22. Elibol-Can B, Simsek-Ozek N, Severcan F, Severcan M, Jakubowska-Dogru E: Vitamin A deficiency induces structural and functional alterations in the molecular constituents of the rat hippocampus. *Br J Nutr,* 113 (1): 45-55, 2015. DOI: 10.1017/s0007114514003432

23. Viccellio P: Systemic poisonous plant intoxication. Handbook of medical toxicology. Washington: Library of congress cataloging. 718, 1993.

24. Popescu R, Kopp B: The genus Rhododendron: An ethno-pharmacological and toxicological review. *J Ethnopharmacol,* 147 (1): 42-62, 2013. DOI: 10.1016/j.jep.2013.02.022

25. Öztaşan N, Altinkaynak K, Akçay F, Göçer F, Dane Ş: Effects of mad honey on blood glucose and lipid levels in rats with streptozocin-induced diabetes. *Turk J Vet Anim Sci,* 29 (5): 1093-1096, 2005.

26. Cakmak-Arslan G, Haksoy H, Goc-Rasgele P, Kekecoglu M: Determination of the dose-dependent toxic effects of mad honey on mouse liver using ATR-FTIR spectroscopy. *Spectrochim Acta A Mol Biomol*

Spectrosc, (Article in Press) 2019. DOI: 10.1016/j.saa.2019.117719

27. de Zwart LL, Meerman JHN, Commandeur JNM, Vermeulen NPE: Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic Biol Med,* 26 (1-2): 202-226, 1999. DOI: 10.1016/s0891-5849(98)00196-8

28. Moore DJ, Sills RH, Mendelsohn R: Peroxidation of erythrocytes: FTIR spectroscopy studies of extracted lipids, isolated membranes, and intact cells. *Biospectroscopy,* 1 (2): 133-140, 1995. DOI: 10.1002/bspy.350010207

29. Lamba OP, Borchman D, Garner WH: Spectral characterization of lipid peroxidation in rabbit lens membranes induced by hydrogen peroxide in the presence of Fe^{2+}/Fe^{3+} cations: A site-specific catalyzed oxidation. *Free Radic Biol Med,* 16 (5): 591-601, 1994. DOI: 10.1016/0891-5849(94)90059-0

30. Barlas S, Tireli E, Dayıoğlu E, Barlas C: Miyokard korunması-II: Miyokard metabolizması ve harabiyeti. *GKD Cer Dergisi,* 2, 313-317, 1994.

31. Manzl C, Enrich J, Ebner H, Dallinger R, Krumschnabel G: Copper-induced formation of reactive oxygen species causes cell death and disruption of calcium homeostasis in trout hepatocytes. *Toxicology,* 196 (1-2): 57-64, 2004. DOI: 10.1016/j.tox.2003.11.001

32. Adam-Vizi V, Starkov AA: Calcium and mitochondrial reactive oxygen species generation: How to read the facts. *J Alzheimers Dis,* 20 (Suppl 2): S413-S426, 2010. DOI: 10.3233/JAD-2010-100465

33. Onat F, Yegen BC, Lawrence R, Oktay A, Oktay S: Site of action of grayanotoxins in mad honey in rats. *J Appl Toxicol,* 11 (3): 199-201, 1991. DOI: 10.1002/jat.2550110308

34. Eraslan G, Kanbur M, Karabacak M, Arslan K, Silig Y, Soyer Sarica Z, Tekeli MY, Tas A: Effect on oxidative stress, hepatic chemical metabolizing parameters, and genotoxic damage of mad honey intake in rats. *Hum Exp Toxicol*, 37, 991-1004, 2018. DOI: 10.1177/0960327117745691

35. Sibel S, Enis YM, Huseyin S, Timucin AA, Duran O: Analysis of grayanatoxin in Rhododendron honey and effect on antioxidant parameters in rats. *J Ethnopharmacol,* 156, 155-161, 2014. DOI: 10.1016/j.jep.2014.08.027

36. Schulze PC, Drosatos K, Goldberg IJ: Lipid use and misuse by the heart. *Circ Res,* 118 (11): 1736-1751, 2016. DOI: 10.1161/circresaha.116.306842

37. Marin-Garcia J, Goldenthal MJ: Fatty acid metabolism in cardiac failure: Biochemical, genetic and cellular analysis. *Cardiovasc Res,* 54 (3): 516-527, 2002. DOI: 10.1016/s0008-6363(01)00552-1

38. Gunduz A, Turedi S, Uzun H, Topbas M: Mad honey poisoning. *Am J Emerg Med,* 24 (5): 595-598, 2006. DOI: 10.1016/j.ajem.2006.01.022

39. Becker W, Kleinsmith L, Hardin J, Wilbur B: Membranes: their structure, function, and chemistry. 158-195, 2003.

40. Megli FM, Sabatini K: Mitochondrial phospholipid bilayer structure is ruined after liver oxidative injury in vivo. *FEBS Lett,* 573 (1-3): 68-72, 2004. DOI: 10.1016/j.febslet.2004.07.057

41. Eken C: Grayanotoksin zehirlenmesi. *Turk J Emerg Med,* 4 (2): 76-77, 2004.

42. Bers DM, Eisner DA, Valdivia HH: Sarcoplasmic reticulum Ca^{2+} and heart failure: Roles of diastolic leak and Ca^{2+} transport. *Circ Res,* 93 (6): 487-490, 2003. DOI: 10.1161/01.Res.0000091871.54907.6b

Kafkas Univ Vet Fak Derg
26 (2): 295-298, 2020
DOI: 10.9775/kvfd.2019.22317

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Short Communication

Anaplasma ovis and *Anaplasma phagocytophilum* Infection in Sheep and Wild Rodents from Northern Xinjiang, Northwest China

Mengmeng JIANG [1,2,†,a] Yan LI [1,†,b] Shichen XIE [1,c] Shengzhong XIAO [1,d] Yanhong ZHANG [1,e]
Yan YANG [1,f] Yuanzhi WANG [2,g] Jinliang SHENG [1,,h]

[†] *These authors contributed equally to this work*
[1] College of Animal Science and Technology of Shihezi University, Shihezi, Xinjiang Uygur Autonomous Region, 832002, P. R. CHINA
[2] School of Medicine, Shihezi University, Shihezi, Xinjiang Uygur Autonomous Region 832002, P. R. CHINA
[a] ORCID: 0000-0003-4119-2478; [b] ORCID: 0000-0002-6566-1366; [c] ORCID: 0000-0002-1922-9695; [d] ORCID: 0000-0002-6002-6355
[e] ORCID: 0000-0003-4377-0446; [f] ORCID: 0000-0002-4389-170X; [g] ORCID: 0000-0002-6002-3124; [h] ORCID: 0000-0003-3420-387X

Article ID: KVFD-2019-22317 Received: 20.03.2019 Accepted: 17.10.2019 Published Online: 23.10.2019

How to Cite This Article

Jiang M, Li Y, Xie S, Xiao S, Zhang Y, Yang Y, Wang Y, Sheng J: *Anaplasma ovis* and *Anaplasma phagocytophilum* Infection in sheep and wild rodents from Northern Xinjiang, Northwest China. *Kafkas Univ Vet Fak Derg,* 26 (2): 295-298, 2020. DOI: 10.9775/kvfd.2019.22317

Abstract

The zoonotic rickettsial pathogen *Anaplasma* species have a broad geographical distribution and are important intracellular agents. Domestic animals and wild rodents may play an important role in the epidemiology of this disease. The aim of this study was to estimate the prevalence of infection with *Anaplasma* species among domestic animals and wild rodents in northern Xinjiang Uygur Autonomous Region (XUAR), Northwest China, during 2015-2016. In this study, DNA from *Anaplasma ovis* was detected by nested PCR in blood samples from 21/137 sheep (15.3%) in Ili Kazakh Autonomous Prefecture (Ili), 18/79 sheep (22.8%) in Bole Mongol Autonomous Prefecture (Bole) and 13/71 sheep (18.3%) in Shihezi City. In addition, detection of *Anaplasma phagocytophilum* DNA in spleen samples from great gerbils *(Rhombomys opimus)* in Urumqi (37/356, 10.4%) and Bole (21/134, 15.7%). Interestingly, co-infection *A. ovis* and *A. phagocytophilum* in blood of sheep (9/137, 6.7%) from Ili, northern XUAR. Based on 16S rRNA sequence, phylogenetic analyses showed that *A. ovis* were separated into one clade, while the *A. phagocytophilum* was separated into another clade. This analysis demonstrated that there are at least two different *Anaplasma* species widespread. The present investigation revealed high infection rates of *A. phagocytophilum* and *A. ovis,* which shed light on making effective measures to prevent and control *Anaplasma* species infection in animals in XUAR, Northwest China.

Keywords: *Anaplasma ovis, Anaplasma phagocytophilum, Prevalence, 16S rRNA, Northwest China, Phylogenetic analyzes*

Kuzeybatı Çin ve Kuzey Sincan'da Koyun ve Yabani Kemirgenlerde *Anaplasma ovis* ve *Anaplasma phagocytophilum* Enfeksiyonu

Öz

Zoonotik rickettsial patojen olan *Anaplasma* türleri geniş bir coğrafi dağılıma sahiptir ve önemli hücre içi ajanlardır. Evcil hayvanlar ve vahşi kemirgenler bu hastalığın epidemiyolojisinde önemli rol oynarlar. Bu çalışmanın amacı, 2015-2016 yıllarında Kuzey Sincan Uygur Özerk Bölgesinde (XUAR), evcil hayvanlar ve vahşi kemirgenler arasında *Anaplasma* türleriyle enfeksiyon prevalansının tespitidir. Bu çalışmada, Ili Kazak Özerk Bölgesi'nde (Ili) 21/137 koyun (%15.3), Bole Mongol Özerk Bölgesi'nde (Bole) 18/79 koyun (%22.8) ve Shihezi Şehrinde 13/71 koyun (%18.3) kan örneğinde yuvalanmış PCR yöntemi ile *Anaplazma ovis* DNA'sı tespit edildi. Ek olarak, Urumçi'deki (37/356, %10.4) ve Bole'deki (21/134, %15.7) büyük rodentlerin *(Rhombomys opimus)* dalak örneklerinde *Anaplasma phagocytophilum* DNA'sı belirlendi. İlginç biçimde, Ili, kuzey XUAR'dan alınan koyunların kanında (9/137, %6.7) *A. ovis* ve *A. phagocytophilum* ile eş zamanlı enfeksiyon mevcuttu. 16S rRNA sekansına dayanarak yapılan filogenetik analizler, *A. ovis*'in bir kola ayrıldığını, *A. phagocytophilum*'un ise başka bir kola ayrıldığını gösterdi. Bu analiz bu bölgelerde en az iki farklı *Anaplasma* türünün yaygın olduğunu göstermiştir. Mevcut araştırma, XUAR, Kuzeybatı Çin'deki hayvanlarda *A. phagocytophilum* ve *A. ovis* enfeksiyon oranlarının yüksek olduğunu ve *Anaplasma* türlerinin enfeksiyonunu önlemek ve kontrol altına almak için etkili önlemler alınmasının gerekliliğini ortaya koydu.

Anahtar sözcükler: *Anaplasma ovis, Anaplasma phagocytophilum, Prevalans, 16S rRNA, Kuzeybatı Çin, Filogenetik analiz*

INTRODUCTION

Xinjiang Uygur Autonomous Region (XUAR) covers 1.66 million square kilometers, which is located in the hinterland of the Eurasian continent is geographically divided into two parts by Tianshan Mountain, namely northern XUAR and southern XUAR [1]. In northern XUAR, which is composed of the desert, Gobi desert, saline beach and

İletişim (Correspondence)
+13 57 9772294
1572621211@qq.com

patched oases. There is a great diversity of tick species in XUAR, owing to the variability of geographical landscape and the availability of multiple vertebrate host species for these parasites. Ticks were reported to transmit bacterial, viral and parasitic diseases to animals and humans [2]. Anaplasmosis is a tick-borne disease and considered emerging or reemerging pathogens with serious public health implications, which are obligate intracellular pathogens that infect humans and animals [3]. Although these agents could infect humans through various routes, animal hosts play an important role in transmission.

Since the first case was reported in the USA in 1990, *Anaplasma* species have been described in both Europe and Asia [4-7]. The major *Anaplasma* species that impact animal and human health include *Anaplasma marginale*, *Anaplasma ovis*, *Anaplasma centrale*, *Anaplasma bovis*, *Anaplasma phagocytophilum*, and *Anaplasma platys* [8]. Among them, *A. phagocytophilum* is distributed worldwide and infects a variety of hosts [9,10]. *A. ovis* is the main inter-erythrocytic pathogens of ovine, which are responsible for ovine anaplasmosis in tropical and subtropical areas [11]. Although studies on *Anaplasma* species have been carried out in part of China [9,12] information is scarce on the animal reservoirs of *Anaplasma* spp. in northern XUAR. Therefore, the aim of this study was to identify *A. ovis* and *A. phagocytophilum* infection in wild and domestic hosts in different districts of northern XUAR, Northwest China

MATERIAL and METHODS

During 2015-2016, blood samples were collected from 137 sheep in Ili Kazakh Autonomous Prefecture (Ili), 79 sheep in Bole Mongol Autonomous Prefecture (Bole) and 71 sheep in Shihezi City. Spleen samples were collected from 356 great gerbils *(Rhombomys opimus)* in Urumqi City and 134 *R. opimus* in Bole in northern XUAR, Northwest China. The study area ranged between latitude 43°49′31.42″N - 44°54′18.04″N and longitude 81°31′27.78″ - 87°36′50.35″E. In accordance with the different types of landscapes, 1-4 sampling sites were selected in each county or city. The sheep bloods were sampled at different intervals under the owner agreements. As to wild rodents (i.e. the great gerbils *(R. opimus)*), their carcasses were submitted for postmortem examination to the Xinjiang Uygur Autonomous Region Wildlife Management Office, and then sent to our laboratory for scientific research. This study was approved by the Animal Ethics Committee of Shihezi University (Approval No. AECSU2015-01).

Genomic DNA was extracted from blood or dissected tissues/organs of the animals (including sheep and the great gerbil) (i.e. spleen in this study) by using the 96 flux automatic nucleic acid extraction instrument with a matching commercial kit (Cell & Tissue Kit, Bioteke, Beijing, China) according to the manufacturer's instructions, cloned into the pBS-T vector, and used for the transformation of One-shot® Top10 *Escherichia coli* cells. To prevent contamination problems, as negative control, used purified sterile water, were tested after every sample in our PCR. To determine genetic variability and regional differences of *A. phagocytophilum* and *A. ovis*, all samples were examined targeting *16S rRNA* genes by polymerase chain reaction (PCR) according to previous descriptions [13,14]. An approximately 400bp fragment was amplified using primers *16S rRNA*-Outer for the first round, then *16S rRNA*-Inner for the second round PCR. Each amplified product was repeatedly sequenced three times. Sequences were compared with GenBank data using BLAST *(http://www.ncbi.nlm.nih.gov/BLAST/)*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [15]. Evolutionary analyses were conducted in MEGA7 [16].

RESUTS

In this study, *A. ovis* was found in the blood samples from sheep in Ili (21/137, 15.3%), Bole (18/79, 22.8%) and Shihezi City (13/71, 18.3%) in northern XUAR, respectively. In addition to the above results, DNA from *A. phagocytophilum* was detected in spleen samples from great gerbils *(R. opimus)* in Urumqi City (37/356, 10.4%) and Bole (21/134, 15.7%), northern XUAR. Interestingly, co-infection *A. ovis* and *A. phagocytophilum* in blood of 9/137 sheep (6.7%) from Ili, northwest China *(Table 1)* was detected.

Based on the analysis of BLAST and phylogenic tree, six *Anaplasma* genotypes *(A. ovis* and *A. phagocytophilum)* were detected *(Fig. 1)*. The sequences of *16S rRNA* fragments amplified from the three *A. ovis* isolates were 100% identical in our study [GenBank No. MK260043, MK260044

Table 1. *Prevalence of Anaplasma ovis and Anaplasma phagocytophilum from northern Xinjiang, northwest China*

District	Host	*Anaplasma ovis* (%)	*Anaplasma phagocytophilum* (%)	Coordinate
Ili	sheep	21/137 (15.3%)	9/137 (6.6%)	43°58′33.93″N 81°31′27.78″E
Bole	sheep	18/79 (22.8%)	-	44°54′18.04″N 82°03′48.45″E
	the great gerbil *(Rhombomys opimus)*	-	21/134 (15.7%)	
Urumqi	the great gerbil *(Rhombomys opimus)*	-	37/356 (10.4%)	43°49′31.42″N 87°36′50.35″E
Shihezi	sheep	13/71 (18.3%)	-	44°18′19.04″N 85°18′19.04″E

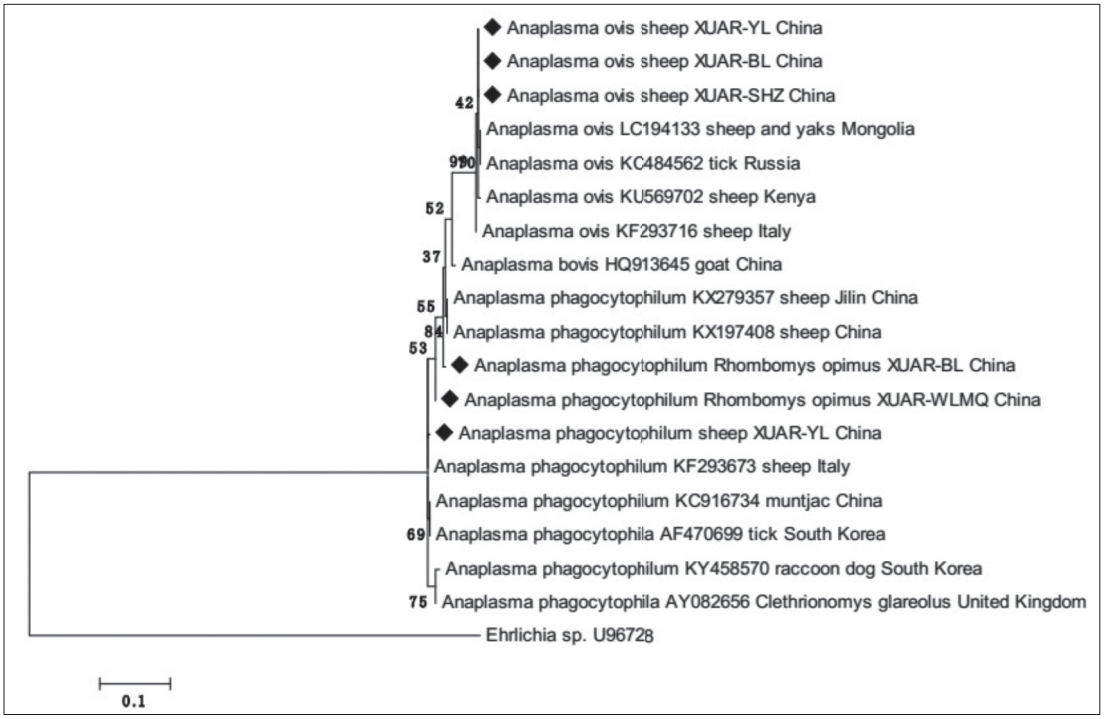


Fig 1. Phylogenetic comparison of *16S rRNA* gene sequence of *A. ovis* and *A. phagocytophilum* (◆) in this study and relevant sequences from GenBank. The results are based on the Maximum-likelihood (ML; 500 bootstrap replicates) approximation of the standard likelihood ratio test score. Branch lengths correlate to the number of substitutions inferred according to the scale shown

and MK260045], and varied from all known *A. ovis* sequences in GenBank, with 99.2%-99.7% nucleotide identity from sheep, yaks and ticks in other countries [GenBank No. KF293716 (Italy), LC194134 (Mongolia), KU569702 (Kenya) and KC484562 (Russia), respectively], and separated into different clusters in the phylogenetic tree. *A. phagocytophilum* from sheep and great gerbils *(R. opimus)* in this work, which had 97.5%-98.8% similarity with the corresponding sequences of *A. phagocytophilum* originated from sheep, muntjac, tick and malebank voles *(Clethrionomys glareolus)* derived from different areas [GenBank No. KX279357 (China), KX197408 (China), KF293673 (Italy), KC916734 (China), AY082656 (United Kingdom), AF470699 and KY458570 (South Korea), respectively]. Interestingly, the sequence divergence between *A. phagocytophilum* from sheep and great gerbils *(R. opimus)* was 2.53% from different regions in our study. The phylogenetic analysis confirmed these results: the separation of genus *Anaplasma* genotypes in the study area were distinct from previously reported in other continents, geographic and host-associated cluster was strongly supported *(Fig. 1)*. All obtained sequences were deposited in GenBank *(A. ovis*: MK260043-MK260045; *A. phagocytophilum*: MK260046-MK260047), but the number has not been released.

DISCUSSION

Since it was first recognised, anaplasmosis caused by *A. ovis* and *A. phagocytophilum* is considered to have a worldwide distribution [4-7]. Studies about the extent of its occurrence in animals and humans have been detected mostly in Europe, the USA and part of Asia [4,6,7]. Here, we isolated four strains of *A. ovis* and *A. phagocytophilum* from sheep, and two strains of *A. phagocytophilum* originated from rodents and used molecular methods to investigate the occurrence of *A. ovis* and *A. phagocytophilum* in northern XUAR, Northwest China.

Ovine anaplasmosis is caused by *A. ovis* in sheep, goats and ticks, which is widely distributed in different regions of the world [1]. The sequences, named as SHZ, YL and BL in XUAR were clustered together, and close to *A. ovis* genotypes from sheep in Kenya and Italy, sheep and yaks in Mongolia and ticks in Russia *(Fig. 1)*. The phylogenetic analysis indicated that *A. ovis* genotype diversity exist for sheep in Italy, Kenya and Mongolia, for yaks in Mongolia and for ticks in Russia, but the geographical differences for each species was not distinguished yet. Although these results add new information on the reservoirs of this disease agent we still need to have more sequence information to identify the differences of every *A. ovis* species.

Anaplasma phagocytophilum is reportedly maintained in various animal reservoirs, such as white-footed mice, goats, sheep, ticks and horses [4,17,18]. The sequence variation in the *16S rRNA* gene among different *A. phagocytophilum* strains confirmed that three genotypes of *A. phagocytophilum* were detected in this study *(Fig. 1)*. *A. phagocytophilum* infection in sheep from Ili compared with another two

A. phagocytophilum genotypes derived from different geographical regions in XUAR showed high diversity to *A. phagocytophilum* of the sheep, raccoon dogs, ticks, *Clethrionomys glareolus* and muntjacs from Italy, South Korea, United Kingdom and other China areas, respectively. These results showed *A. phagocytophilum* genotypes and displayed a high degree of genetic diversity, geographical and host tropisms, and the results coincides with Barakova et al.[19] report. To determine the level of infectivity in rodents as well as domestic animals, further studies are needed.

In northern XUAR, tick species distributed widespread, it is important to mention that animals located in this area suffer from heavy infestations by ticks. Here, we concluded that the emerging tick-borne *A. ovis* and *A. phagocytophilum* infection is already prevalent in different areas of ChinaThese results thus add new information on the reservoirs of those disease agents. Despite the existence of the biggest livestock industry in northwest China, there is still a considerable gap in our knowledge regarding the distribution of these pathogens and its economical relevance. In the future, it is important that tick-borne *Anaplasma* species involving domestic animals, wildlife, and humans should be paid more attention to the cooperation of Central Asia countries.

Acknowledgment

This research was supported in part by the National key Research & Development Program of China (Grant No. 2017YFD0500301, 2017YFD0500304), and the Xinjiang Bingtuan Science and Technology Development Fund (2017BA044).

Conflict of Interest

These authors have no conflict of interest related with this study.

REFERENCES

1. Song R, Wang Q, Guo F, Liu X, Song S, Chen C, Tu C, Wureli H, Wang Y: Detection of *Babesia* spp. *Theileria* spp. and *Anaplasma ovis* in border regions, northwestern China. *Transbound Emerg Dis*, 65 (6): 1537-1544, 2018. DOI: 10.1111/tbed.12894

2. Sheng J, Jiang M, Yang M, Bo X, Zhao S, Zhang Y, Hazihan W, Wang B, Tu C, Wang Y: Tick distribution in border regions of Northwestern China. *Ticks Tick Borne Dis*, 10 (3): 665-669, 2019. DOI: 10.1016/j.ttbdis.2019.02.011

3. Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR: Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order Rickettsiales: Unification of some species of *Ehrlichia* with *Anaplasma*, Cowdria with *Ehrlichia* and *Ehrlichia* with Neorickettsia, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol*, 51, 2145-2165, 2001. DOI: 10.1099/00207713-51-6-2145

4. Wormser GP: Accuracy of diagnosis of human granulocytic Anaplasmosis in China. *Emerg Infect Dis,* 22 (10): 1728-1731, 2016. DOI: 10.3201/eid2210.160161

5. Chen SM, Dumler JS, Bakken JS, Walker DH: Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. *J Clin Microbiol*, 32 (3): 589-595, 1994.

6. Dumler JS, Choi KS, Garcia-Garcia JC, Barat NS, Scorpio DG, Garyu JW, Grab DJ, Bakken JS: Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. *Emerg Infect Dis,* 11, 1828-1834, 2005. DOI: 10.3201/eid1112.050898

7. Fourie JJ, Evans A, Labuschagne M, Crafford D, Madder M, Pollmeier M, Schunack B: Transmission of *Anaplasma phagocytophilum* (Foggie, 1949) by *Ixodes ricinus* (Linnaeus, 1758) ticks feeding on dogs and artificial membranes. *Parasit Vectors*, 12:136, 2019. DOI: 10.1186/s13071-019-3396-9

8. Liu Z, Ma M, Wang Z, Wang J, Peng Y, Li Y, Guan G, Luo J, Yin H: Molecular survey and genetic identification of *Anaplasma* species in goats from central and southern China. *Appl Environ Microbiol*, 78 (2): 464-470, 2012. DOI: 10.1128/AEM.06848-11

9. Yang J, Liu Z, Guan G, Liu Q, Li Y, Chen Z, Ma M, Liu A, Ren Q, Luo J, Yin H: Prevalence of *Anaplasma phagocytophilum* in ruminants, rodents and ticks in Gansu, north-western China. *J Med Microbiol*, 62, 254-258, 2013. DOI: 10.1099/jmm.0.046771-0

10. Perez G, Bastian S, Chastagner A, Agoulon A, Plantard O, Vourc'h G, Butet A: Ecological factors influencing small mammal infection by *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l. in agricultural and forest landscapes. *Environ Microbiol*, 19 (10): 4205-4219, 2017.

11. de la Fuente J, Atkinson MW, Naranjo V, Fernández de Mera IG, Mangold AJ, Keating KA, Kocan KM: Sequence analysis of the msp4 gene of *Anaplasma ovis* strains. *Vet Microbiol*, 119 (2-4): 375-381, 2007.

12. Zhan L, Cao WC, Chu CY, Jiang BG, Zhang F, Liu W, Dumler JS, Wu XM, Zuo SQ, Zhang PH, Huang HN, Zhao QM, Jia N, Yang H, Richardus JH, Habbema JDF: Tick-borne agents in rodents, China, 2004-2006. *Emerg Infect Dis*, 15 (12): 1904-1908, 2009. DOI: 10.3201/eid1512.081141

13. Liu Z, Luo J, Bai Q, Ma M, Guan G, Yin H: Amplification of 16S rRNA genes of *Anaplasma* species in China for phylogenetic analysis. *Vet Microbiol*, 107 (1-2): 145-148, 2005. DOI: 10.1016/j.vetmic.2005.01.007

14. Zhang L, Wang G, Liu Q, Chen C, Li J, Long B, Yu H, Zhang Z, He J, Qu Z, Yu J, Liu Y, Dong T, Yao N, Wang Y, Cheng X, Xu J: Molecular analysis of *Anaplasma phagocytophilum* isolated from patients with febrile diseases of unknown etiology in China. *Plos One*, 8 (2): e57155, 2013. DOI: 10.1371/journal.pone.0057155

15. Tamura K, Nei M: Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, 10 (3): 512-526, 1993. DOI: 10.1093/oxfordjournals.molbev.a040023

16. Kumar S, Stecher G, Tamura K: MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*, 33 (7): 1870-1874, 2016. DOI: 10.1093/molbev/msw054

17. Ravyn MD, Kodner CB, Carter SE, Jarnefeld JL, Johnson RC. Isolation of the etiologic agent of human granulocytic *Ehrlichiosis* from the white-footed mouse *(Peromyscus leucopus). J Clin Microbiol*, 39 (1): 335-338, 2001.

18. Scorpio DG, Leutenegger C, Berger J, Barat N, Madigan JE, Dumler JS: Sequential analysis of *Anaplasma phagocytophilum msp2* transcription in murine and equine models of human granulocytic anaplasmosis. *Clin Vaccine Immunol*, 15 (3): 418-424, 2008. DOI: 10.1128/CVI.00417-07

19. Barakova I, Derdakova M, Carpi G, Rosso F, Collini M, Tagliapietra V, Ramponi C, Hauffe HC, Rizzoli A: Genetic and ecologic variability among *Anaplasma phagocytophilum* strains, northern Italy. *Emerg Infect Dis*, 20 (6): 1082-1084, 2014. DOI: 10.3201/eid2006.131023

Kafkas Univ Vet Fak Derg
26 (2): 299-303, 2020
DOI: 10.9775/kvfd.2019.22661

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Short Communication

The End-systolic Wall Stress/End-systolic Volume Index Ratio for Systolic Function in Anatolian Shepherd Dogs with Stage B2 Degenerative Mitral Valve Disease [1]

Kürşad TURGUT [1,a] İsmail ŞEN [2,b] Mehmet Ege İNCE [1,c] Amir NASERİ [3,d]
Havva SÜLEYMANOĞLU [1,e] Merve ERTAN [1,f] Vedat SAĞMANLIGİL [4,g]

[1] *Oral presentation at congress: 32nd World Veterinary Congress (WVC), 13-17 September 2015 - Istanbul, Turkey*
[1] Near East University, Faculty of Veterinary Medicine, Department of Internal Medicine, 99280, Nicosia, NORTH CYPRUS
[2] Kyrgyz Turkish Manas University, Faculty of Veterinary Medicine, Department of Internal Medicine, 720011 Bishkek - KYRGYZSTAN
[3] Selcuk University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-42003 Konya - TURKEY
[4] Near East University, Faculty of Veterinary Medicine, Department of Physiology, 99280, Nicosia, NORTH CYPRUS
[a] ORCID: 0000-0001-8725-8044; [b] ORCID: 0000-0002-2965-7183; [c] ORCID: 0000-0002-1859-1449; [d] ORCID: 0000-0001-9140-5879
[e] ORCID: 0000-0002-6681-0212; [f] ORCID: 0000-0003-3436-4817; [g] ORCID: 0000-0001-9335-7348

Article ID: KVFD-2019-22661 Received: 17.05.2019 Accepted: 17.09.2019 Published Online: 17.09.2019

How to Cite This Article

Turgut K, Şen İ, İnce ME, Naseri A, Süleymanoğlu H, Ertan M, Sağmanligi V: The end-systolic wall stress/end-systolic volume index ratio for systolic function in Anatolian shepherd dogs with stage B2 degenerative mitral valve disease. *Kafkas Univ Vet Fak Derg,* 26 (2): 299-303, 2020. DOI: 10.9775/kvfd.2019.22661

Abstract

This study was performed to evaluate the end-systolic wall stress/end-systolic volume index ratio (ESWS/ESV-I) for LV systolic function in Anatolian shepherd dogs (ASHs) with stage B2 degenerative mitral valve disease (DMVD). Thirty-eight adult ASHs with DMVD (experimental group; 20 B1 dogs and 18 B2 dogs) and 30 weight-matched adult healthy ASHs (control group) were used as subjects. There was no overlap for the ESWS/ESV-I ratio (0.83±0.03, range: 0.61-1.09 for B2 dogs, 1.34±0.05, range: 1.11-1.89 for B1 dogs, 2.17±0.0 range: 1.91-3.02 for the control dogs) among the groups. ESWS/ESV-I ratio (≤1.09) in ASHs with stage B2 DMVD may play a developmental role in left ventricular remodelling and risk factor for development of systolic dysfunction.

Keywords: *Anatolian shepherd dog, Degenerative mitral valve disease, Echocardiography, End-systolic wall stress/end-systolic volume index ratio, Systolic function indices*

Evre B2 Dejeneratif Mitral Kapak Hastalığı Olan Anadolu Çoban Köpeklerinde Sistol-sonu Duvar Stresi/Sistol-sonu Hacim İndeks Oranı İle Sistolik Fonksiyonun Değerlendirilmesi

Öz

Bu çalışma, evre B2 dejeneratif mitral kapak hastalığı (DMVD) olan Anadolu çoban köpeklerinde (ASHs) sistol-sonu duvar stresi/sistol-sonu hacim indeks oranı (ESWS/ESV-I) ile sistolik fonksiyonu değerlendirmek için yapıldı. Çalışmada, DMVD'li 38 yetişkin ASHs (deney grubu; 20 B1 köpek ve 18 B2 köpek) ve 30 yetişkin sağlıklı ASHs (kontrol grubu) kullanıldı. Gruplar arasında ESWS/ESV-I oranı için (B2 köpeklerinde: 0.83±0.03, aralık: 0.61-1.09, B1 köpeklerinde 1.34±0.05, aralık: 1.11-1.89, kontrol köpeklerinde 2.17±0.0 aralık: 1.91-3.02) bir örtüşme yoktu. Evre B2 DMVD'li ASH'lerde, ESWS/ESV-I oranı (≤1.09) sol ventrikül yeniden yapılanması ve sistolik disfonksiyon gelişiminde risk faktörü olarak rol oynayabilir.

Anahtar sözcükler: *Anadolu çoban köpeği, Dejeneratif mitral kapak hastalığı, Ekokardiyografi, Sistol-sonu duvar stresi/Sistol-sonu hacim indeksi oranı, Sistolik fonksiyon endeksleri*

INTRODUCTION

The most common heart diseases causing heart failure (HF) in dogs is degenerative mitral valve disease (DMVD) [1,2]. Dogs in stage B2 may be days away from developing congestive heart failure (CHF), or may never develop CHF in their life time [2]. Systolic function is not easy to evaluate in DMVD because of decreased afterload, increased preload

İletişim (Correspondence)
+90 533 8594430
kursad.turgut@neu.edu.tr

and enhanced sympathetic tone [3,4]. Therefore, systolic function indices, such as fractional shortening (FS), end-systolic volume index (ESV-I) and E point septal separation (EPSS) remain high or no change during the compensated state of chronic mitral regurgitation (MR), despite reduced myocardial functioning [5].

Many authors have advocated the use of different parameters in dealing with wall stress to better define left ventricular function in humans. Alter et al.[6] suggested that elevated end-diastolic wall stress (EDVS) was a strong predictor of systolic and diastolic dysfunction. Clerfond et al.[7] informed that end-systolic wall stress (ESWS) was an independent predictor of LV remodelling and post-discharge HF after myocardial infarction (MI). Carabello et al.[8] suggested that the end-systolic wall stress/end-systolic volume index ratio (ESWS/ESV-I) might be helpful in assessing left ventricular systolic function and operative risk in human patients with chronic, symptomatic MR.

End systolic volume (ESV) is independent of preload and has been shown to vary linearly with afterload. Afterload has been approximated by measuring either end-systolic pressure or ESWS [3]. The importance of ESWS/ESV-I ratio and its applicability are unknown in dogs with DMVD. Our hypothesis is that ESWS/ESV-I ratio is related with ventricular function and thus provides a new criterion in determining LV remodelling and systolic function. It seemed logical to us to determine if the ESWS/ESV-I ratio would be higher (indicating relatively greater left ventricular shortening) or would be lower (indicating less shortening and thus relatively poorer left ventricular function). For this reason, our study aimed to assess the ESWS/ESV-I ratio for LV systolic function in Anatolian shepherd dogs (ASHs) with asymptomatic DMVD.

MATERIAL and METHODS

Legal Conformity and Ethics Statement

Ethical approval was received from the Faculty of Veterinary Medicine of Selçuk University (permit number 2012/053).

Animals

This retrospective study used 38 adult (age: median 5.87 (3-9) years; weight: 41±3.29 (28-55) kg gender: 31 males, 7 females) ASHs with DMVD (experimental group) and 30 weight-matched, adult (age: median 5.17 (2-9) years; weight: 40±6.33 (27-52) kg gender: 25 males, 5 females), healthy ASHs (control group) as subjects. All the ASHs had been presented for a cardiology consultation both for the identification of a heart murmur and for the evaluation of the cardiovascular system or for the regular checkup purpose.

Study Design and Clinical Examination

A clinical examination, an electrocardiography (ECG) (Vet ECG Electrocardiograph VE-300, Vega Group) study, thoracic radiography for VHS determination [3] and arterial blood pressure (BP, systolic and diastolic) measurements using oscillometric technique (DynaPulse DP 5200A Pathway Pulse Dynamic NIBP Monitor, Pulse Metric Inc., USA) [3] were done in each ASH for both the experimental and control groups. The intensity of cardiac murmurs was evaluated according to Levine's classification [3]. NT-proBNP concentrations (CardioPet, NT-proBNP, IDEXX, Westbrook, ME) were measured by enzyme immunoassay (EIA).

Each dog was examined using a standard 2-D, M-mode, and colour-flow Doppler imager.

The presence of ticked valve leaflets and/or prolapsed and the identification of MR was inclusion criteria in the experimental group. The control dogs had no symptoms of heart disease showing normal blood pressure, chest radiography, ECG, and echocardiographic findings. Dogs with any medical treatment for mitral valve disease or that had extra-cardiac disease based on history, physical examination, and a chemistry profile was excluded.

The degree of heart disease was classified according to the ACVIM consensus statement [9], according to radiographic and echocardiographic heart size. In order to differentiate class B animals as B1 and B2, the vertebral heart scale (VHS) and echocardiographic left atrial/aortic root (LA/Ao) ratio were performed. From the experimental group, 18 dogs had both VHS scores >10.5 vertebrae and LA/Ao ratios >1.7 (stage B2), whereas 20 had both VHS scores ≤10.5 vertebrae and LA/Ao ratios ≤1.7 (stage B1).

Echocardiography

Transthoracic echocardiographic examinations (2-D, M-mode and Doppler) were performed in all dogs according to the techniques described by Turgut [3]. Transducer arrays of 4-7 MHz were used (SUIU, CZXL-43C).

The regurgitant jet area signal to the LA area (ARJ/LAA) and the LA/Ao ratio were classified as described by Turgut [3]. M-mode measurements including EPSS, LV end-diastolic diameter (D_{ed}), end-systolic diameter (D_{es}), IVS_{ed} and LV posterior wall thickness in end-systole (PWh_{es}) were obtained according to the leading-edge-to-leading-edge method. The FS, end-diastolic volume (EDV), ESV and stroke volume were calculated using the Teichholz method using the software program of the echocardiogram. The EDV, ESV and cardiac output values were indexed for body surface area. This was in order to obtain the EDV-I, ESV-I and the cardiac index (CI).

IVSd/LVd ratio; The IVSd/LVd ratio was determined by M-mode measurements from right parasternal long-axis 5-chamber view.

ESWS/ESV-I ratio; The ESWS is determined by the ratio of left ventricular internal dimension (D_{es}) to the posterior wall thickness (PWh_{es}):

ESWS = (PxD$_{es}$) × 1.35/PWh$_{es}$; where ESWS is left ventricular wall stress (g/cm^2) at end-systole, P is the systolic BP (mmHg), D$_{es}$ and PWh$_{es}$ are the left ventricular internal dimension and posterior wall thickness (cm) at end-systole, respectively, and 1.35 is a conversion factor (mmHg to g/cm^2). Thus, the determination of ESWS and ESV-I allowed for calculation of the ESWS/ESV-I ratio.

Statistical Methods

All data (control, B1, and B2 groups) were evaluated by a homogenity test (Kolmogrov-Smirnov Z) to determine the data distributions. The values for FS, EPSS, NT-proBNP and LA/Ao were evaluated using Mann-Whitney U test, because of the data's nonparametric distribution. Values for FS, EPSS, NT-proBNP and LA/Ao are reported as the median. The values for VHS, ESV-I, CI, EDV-I, the IVSd/LVd ratio and the ESWS/ESV-I ratio were evaluated using a One-way ANOVA and Tukey post hoc test (SPSS 19.0). The values for VHS, ESV-I, CI, EDV-I, the IVSd/LVd ratio and the ESWS/ESV-I ratio are reported as the mean ± SE. The statistical significance level was set at P<0.05.

RESULTS

On the physical examination, 16 (80%) of the B1 dogs had left-apical holosystolic murmurs (13 with grade I-II/VI; 3 with grade III-IV/VI) and 4 (20%) had no an auscultable murmur. 17 (94.4%) of the B2 dogs had left-apical holosystolic murmurs (10 with grade I-II/VI; 7 with grade III-IV/VI) and 1 (5.6%) had no an auscultable murmur.

The left ventricular systolic function parameters (FS, ESV-I, EPSS and ESWS/ESV-I ratio), EDV-I, NT- proBNP, VHS, LA/Ao, IVSd/LVd and ESWS/ESV-I values in the control and the experimental (B1 and B2) groups of dogs are summarised in *Table 1*.

On the colour-flow Doppler examination, all 20 B1 dogs (100%) had mild MR (<20%), whereas among 18 B2 dogs, 6 (33%) had mild, 11 (61%) had moderate (20-40%) *(Fig. 1)* and 1 (6%) had severe (>50%) MR.

The mean ESWS/ESV-I ratio was (*P*<0.05) lower in the B2 dogs (0.83±0.03, range: 0.61-1.09) when compared with the B1 dogs (1.34±0.05, range: 1.11-1.89) and control dogs (2.17±0.0, range: 1.91-3.02). The difference between the B2 dogs and the B1 dogs *(Table 1, Fig. 2)* was also significant (P<0.05).

DISCUSSION

Many studies have provided important information on the natural progression of stage B2 DMVD and reported factors that can be used to identify which stage B2 dogs have higher versus lower risks of developing CHF [10,11]. The results of studies on the effect of volume overload caused by DMVD on left ventricular function are controversial [2,4,8]. In our study, FS, ESV-I, CI showed a significant difference, especially in the B2 dogs *(Table 1)*. However, there was some overlap for FS (29-45% for the control dogs, 31-48% for theB1 dogs, 45-51% for the B2 dogs) *(Table 1)*, for the ESV-I (5.84-30.16 mL/m^2 for the control dogs, 13.69-42.59 mL/m^2 for the B1 dogs, 22.37-36.23 mL/m^2 for the B2 dogs *(Table 1)*, for the CI (2907-6399 ml/min/m^2 for the control dogs, 1267-5285 mL/min/m^2 for the B1 dogs, 2112-4304 mL/min/m^2 for the B2 dogs) among the groups, and these indexes did not manage to separate the groups *(Table 1)*. This might be the result of decreased afterload, increased preload and sympathetic tone. For these reasons, we think that FS, ESV-I and CI are thought to be less sensitive indicators of systolic function in asymptomatic ASHs with DMVD, which is in agreement with previous studies [4,12]. All these results may demonstrate that there seemed to be

Table 1. *NT- proBNP, VHS, LA/Ao, LV, FS, ESV-I, CI, EPSS, EDV-I, IVSd/LVd and ESWS/ESV-I ratio in the control and experimental groups of dogs*

Paramaters	Control group (n = 30)	Experimental group	
		B1 (n = 20)	B2 (n = 18)
NT- proBNP (pmol/L) (median and range)	759±47 (250-2050)^c	1588±212 (900-4650)^b	2985±218 (1250-4300)^a
VHS vertebrae (mean and range)	9.74±0.06 (9.10-10.50)^c	10.30±0.09 (9.00-10.50)^b	10.90±0.05 (10.60-11.50)^a
LA/Ao (median and range)	1.20±0.03 (0.69-1.58)^c	1.44±0.04 (1.10-1.70)^b	1.80±0.03 (1.73-2.10)^a
FS (%) (median and range)	35.00 (29-45)^c	44.50 (31-48)^b	46.50 (45-51)^a
ESV-I (mL/m^2) (mean and range)	17.1±1.39 (5.84-30.16)^b	27.6±2.35 (13.69-42.59)^a	31.7±1.0 (22.37-36.23)^a
CI (mL/min/m^2) (mean and range)	4352±186 (2907-6399)^a	3227±226 (1267-5285)^a	3165±157 (2112-4304)^b
EPSS (mm) (median and range)	3.70 (2.00-6.50)	3.40 (2.30-7.00)	3.35 (2.50-4.40)
EDV-I (mL/m^2) (mean and range)	55.7±2.4 (29.9-78.1)^b	52.2±3.6 (31.4-73.7)^b	75.7±2.2 (54.2-85.2)^a
IVSd/LVd(mean and range)	0.27±0.007 (0.22-0.33)	0.28±0.008 (0.22-0.33)	0.27±0.01 (0.23-0.33)
ESWS/ESV-I ratio (mean and range)	2.17±0.0 (1.91-3.02)^a	1.34±0.05 (1.11-1.89)^b	0.83±0.03 (0.61-1.09)^c

FS: *fractional shortening;* ***EF:*** *ejection fraction;* ***ESV-I:*** *end-systolic volume index;* ***EPSS:*** *E point septal separation;* ***EDV-I:*** *end-diastolic volume index;* ***CI:*** *cardiac index;* ***ESWS/ESV-I:*** *end-systolic wall stress/end-systolic volume ratio;* ***IVSd/LVd:*** *interventricular septum thickness/left ventricular internal dimension ratio;* ^{a,b,c} *Values with different character in each row are statistically different with P<0.05*

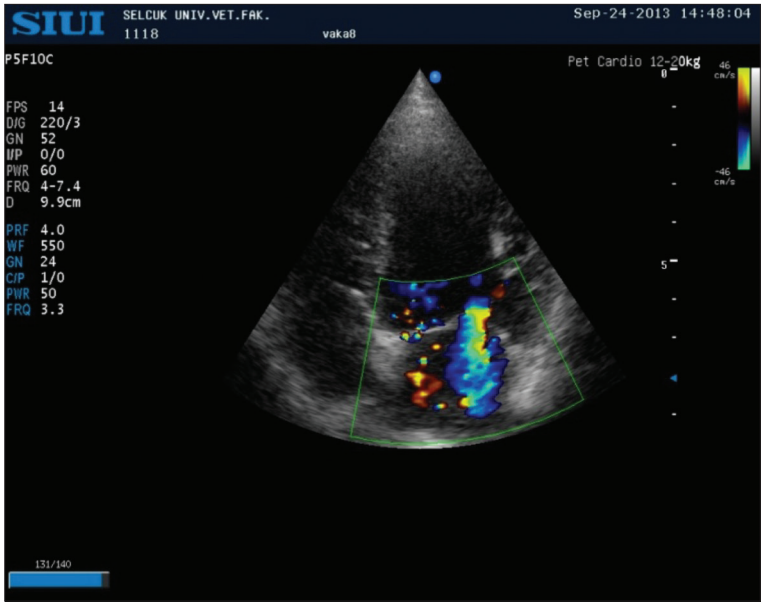
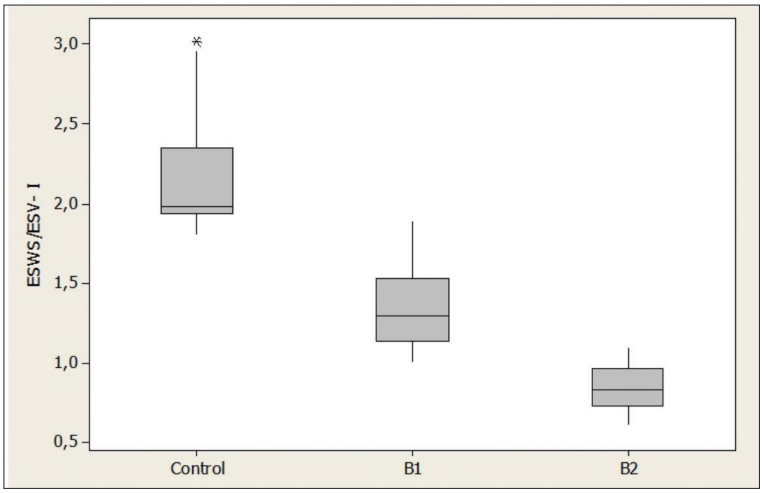


Fig 1. Moderate mitral regurgitation jet in a B2 dog with DMVD

Fig 2. ESWS/ESV-I ratio distribution for the control dogs, B1 dogs, and B2 dogs. There was no overlap for the ESWS/ESV-I ratio among the groups, and the ESWS/ESV-I ratio did separate the groups alone



preserved systolic function and a hyper-dynamic phase of the heart in the B1 and B2 dogs.

Chamber enlargement may lead to increased peak systolic wall stress (by the Law of La Place), which causes wall thickening of a sufficient magnitude to normalise the systolic stress [3]. Suzuki et al.[13], have also reported that relative wall thickness was not a factor in determining asymptomatic dogs with Class I DMVD and healthy dogs.

Increased ESWS in LV volume overload causes mainly chamber enlargement, and eccentric hypertrophy. ESWS probably gives more accurate prediction when determining afterload than using pressure alone, because it deals with filling conditions, BP, wall thickness and curvature radius [7]. Clerfond et al.[7] informed that ESWS was an independent estimator of LV remodelling and post-discharge HF after MI. When we consider ESV-I, dogs with ESV-I values>30 mL/m^2 indicate impaired systolic function [3]. In our study, ESV-I was high in the B2dogs (>30 mL/m^2) *(Table 1)*. For this reason we thought ESWS/ESV-I would be useful in evaluating systolic function. Carabello et al.[8] studied patients with symptomatic, chronic, severe MR to determine which hemodynamic and angiographic factors might be prognostic of surgical outcome. They found that the ESWS/ESV-I ratio was more sensitive than EF or other hemodynamic variables in predicting surgical outcome in symptomatic patients with chronic MR. In our study, the mean ESWS/ESV-I ratio was lower (P<0.05) in the B2 dogs (0.83±0.03, range: 0.61-1.09) when compared with B1 dogs (1.34±0.05, range: 1.11-1.89) and control dogs (2.17±0.0 range: 1.91-3.02). The difference between the B2 dogs and the B1 dogs was also significant (P<0.05). There was no overlap for the ESWS/ESV-I ratio among the groups, and the ESWS/ESV-I ratio did separate the groups alone *(Table 1, Fig. 2)*. All the ESWS/ESV-I ratios were ≤1.09 for the B2 group. This may indicate that the ESWS/ESV-I ratio is more accurate in assessing left ventricular function. Thus, the B2 dogs, who had a median FS of 46.50% (range: 45-51%), had a significantly lower ESWS/ESV-I ratio (≤1.09) than both control dogs and B1 dogs, indicating left ventricular function. The level of

NT-proBNP secreted predominantly from the ventricle is elevated in response to cardiac remodelling [14,15]. In our study, NT-proBNP levels (the B2 dogs: 2985±218 pmol/L (1250-4300 pmol/L), the B1 dogs: 1588±212 pmol/L (900-4650 pmol/L) and the control dogs: 759±47 pmol/L (250-2050 pmol/L) were irrespectively correlated with ESWS/ESV-I ratio *(Table 1)*. These results could be the reason of synthesis and secretion of NT-proBNP induced by the elevation of left ventricular ESWS in the B1 and the B2 dogs. It may suggest that the echocardiography-based approach could be improved through the determination of BNP.

Our results highlight that ESWS/ESV-I ratio (≤1.09) in ASHs with stage B2 DMVD may result in left ventricular remodelling and a risk factor in the development of systolic dysfunction; in other words, it may be to foresee the transition from asymptomatic to a symptomatic DMVD in dogs.

Acknowledgments

This study was financed by the Scientific and Technological Research Council of Turkey (TÜBİTAK). Project No: 1120908

REFERENCES

1. Menciotti G, Borgarelli M: Review of diagnostic and therapeutic approach to canine myxomatous mitral valve disease. *Vet Sci,* 4:47, 2017. DOI: 10.3390/vetsci4040047

2. Gordon SG, Saunders AB, Wesselowski SR: A symptomatic canine degenerative valve disease: Current and future therapies. *Vet Clin North Am Small Anim Pract,* 47, 955-975, 2017. DOI: 10.1016/j.cvsm.2017.04.003

3. Turgut K: Klinik Kedi ve Köpek Kardiyolojisi. Nobel Tıp Kitapevleri. İstanbul, Türkiye, 2017.

4. Petric AD: Myxomatous mitral valve disease in dogs - An update and perspectives. *Mac Vet Rev,* 38 (1): 13-20, 2015. DOI: 10.14432/j.macvetrev.2014.11.026

5. Chetboul V, Tissier R: Echocardiographic assessment of canine degenerative mitral valve disease. *J Vet Cardiol*, 14, 127-148, 2012. DOI: 10.1016/j.jvc.2011.11.005.

6. Alter P, Koczulla AR, Nell C, Figiel JH, Vogelmeier CF, Rominger MB: Wall stress determines systolic and diastolic function - Characteristics of heart failure. *Int J Cardiol*, 202, 685-693, 2016. DOI: 10.1016/j.ijcard.2015.09.032

7. Clerfond G, Bière L, Mateus V, Grall S, Willoteaux S, Prunier F, Furber A: End-systolic wall stress predicts post-discharge heart failure after acute myocardial infarction. *Arch Cardiovasc Dis,* 108, 310-320, 2015. DOI: 10.1016/j.acvd.2015.01.008

8. Carabello BA, Stanton P, Nolan MD, Lockhart B, Mcguire MD: Assessment of preoperative left ventricular function in patients with mitral regurgitation: Value of the end-systolic wall stress-end-systolic volume ratio. *Circulation*, 64, 1212-1217, 1981.

9. Matos JM, Glaus TM: Medical treatment of canine heart failure. *EJCAP,* 20, 171-176, 2010.

10. Reynolds CA, Brown DC, Rush JE, Fox PR, Nguyenba TP, Lehmkuhl LB, Gordon SG, Kellihan HB, Stepien RL, Lefbom BK, Meier CK, Oyama MA: Prediction of first onset of congestive heart failure in dogs with degenerative mitral valve disease: the PREDICT cohort study. *J Vet Cardiol*, 14 (1): 193-202, 2012. DOI: 10.1016/j.jvc.2012.01.008

11. Mattin MJ, Boswood A, Church DB, Lopez-Alvarez J, McGreevy PD, O'Neill DG, Thomson PC, Brodbelt DC: Prevalence of and risk factors for degenerative mitral valve disease in dogs attending primary-care veterinary practices in England. *J Vet Intern Med,* 29, 847-854, 2015. DOI: 10.1111/jvim.12591

12. Chetboul V, Carlos Sampedrano C, Gouni V, Nicolle AP, Pouchelon JL, Tissier R: Ultrasonographic assessment of regional radial and longitudinal systolic function in healthy awake dogs. *J Vet Intern Med,* 20, 885-893, 2006. DOI: 10.1111/j.1939-1676.2006.tb01802.x

13. Suzuki R, Matsumoto H, Teshima T, Mochizuki Y, Koyama H: Left ventricular geometrical differences in dogs with various stages of myxomatous mitral valve disease. *J Small Anim Pract,* 54, 234-239, 2013. DOI: 10.1111/jsap.12058

14. Fox PR, Oyama MA, Hezzell, MJ, Rush JE, Nguyenba TP, DeFrancesco TC, Lehmkuhl LB, Kellihan HB, Bulmer B, Gordon SG, Cunningham SM, MacGregor J, Stepien RL, Lefbom B, Adin D, Lamb K: Relationship of plasma N-terminal pro-brain natriuretic peptide concentrations to heart failure classification and cause of respiratory distress in dogs using a 2nd generation ELISA assay. *J Vet Intern Med,* 29, 171-179, 2014. DOI: 10.1111/jvim.12472

15. Strunz CMC, Marcondes-Santos M, Takada JY, Fragata FS, de Padua Mansur A: Quality of life score as a predictor of death in dogs with degenerative mitral valve disease. *Arq Bras Cardiol,* 108 (4): 347-353, 2017. DOI: 10.5935/abc.20170032

Kafkas Univ Vet Fak Derg
26 (2): 305-308, 2020
DOI: 10.9775/kvfd.2019.22890

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Short Communication

Characterisation of Genital *Mycoplasma* Species from Preputial Swabs of Bucks and Rams [1]

Hüban GÖÇMEN [1,a] Selim ALÇAY [2,b] Ruben S. ROSALES [3,c] Anne RIDLEY [4,d]

[1] *This research was presented in the International Human and Animal Mycoplasmology Congress 3-5 June 2015, Istanbul - Turkey*

[1] Department of Microbiology, Faculty of Veterinary Medicine, Near East University, Mersin-10, 99138, Nicosia, Northern Cyprus, TURKEY
[2] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Uludag University, TR-16059 Bursa - TURKEY
[3] Institute for Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria, C/Trasmontaña s/n, Arucas, 35416, Gran Canaria, SPAIN
[4] Animal and Plant Health Agency (Weybridge), Department of Bacteriology, New Haw, Addlestone, Surrey, KT15 3NB, UNITED KINGDOM
[a] ORCID: 0000-0002-2245-5781; [b] ORCID:0000-0002-2472-8157; [c] ORCID: 0000-0003-3706-0181; [d] ORCID: 0000-0001-6780-0609

Article ID: KVFD-2019-22890 Received: 25.06.2019 Accepted: 22.11.2019 Published Online: 22.11.2019

How to Cite This Article

Göçmen H, Alçay S, Rosales RS, Ridley A: Characterisation of genital *Mycoplasma* species from preputial swabs of bucks and rams. *Kafkas Univ Vet Fak Derg,* 26 (2): 305-308, 2020. DOI: 10.9775/kvfd.2019.22890

Abstract

Mycoplasma species are a major cause of mastitis, arthritis, pneumonia and reproductive disorders in small ruminants. Mycoplasmas in reproductive systems of males have been associated with diseases as orchitis, balanoposthitis and abnormal spermatozoa activity. The aim of this study was to detect *Mycoplasma* species that cause reproductive infections in bucks and rams. Total of 27 preputial swabs was collected from Saanen bucks and Kıvırcık rams at the Artificial Insemination (AI) Center of Uludag University in Turkey. Bacteriological culture methods, followed by Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE) were used to detect and identify *Mycoplasma* species. The PCR-DGGE method identified one *M. bovigenitalium* and one *M. arginini* from two cases of orchitis in rams, and another *M. bovigenitalium* was identified from a buck with no clinical signs. The results showed that *Mycoplasma* species were present in the testicles of rams and bucks that were negative for Brucellosis, and likely causative organisms of orchitis which lead to reduced fertility.

Keywords: *Genital Mycoplasmas, PCR-DGGE, Preputial swab, Small ruminant*

Teke ve Koçların Prepusyal Svap Örneklerinden Genital Mikoplazma Türlerinin Karakterizasyonu

Öz

Mikoplazma türleri küçük ruminantlarda mastitis, artritis, pnömoni ve reprodüktif bozukluklara neden olabilmektedir. Erkek hayvanların reprodüktif sistemlerinde bulunan mikoplazmalar; orşitis, balanopostitis ve anormal spermatozoa aktivitesi gibi hastalıklarla ilişkilendirilmiştir. Bu çalışmada, teke ve koçlarda reprodüktif infeksiyonlara neden olan mikoplazma türlerinin tespiti ve karakterizasyonu amaçlandı. Uludağ Üniversitesi Suni Tohumlama Merkezi'nde bulunan Saanen ırkı tekelerden ve Kıvırcık ırkı koçlardan toplamda 27 adet prepusyal svap örnekleri toplandı. Mikoplazma türlerinin izolasyon ve identifikasyonu için bakteriyolojik kültür yöntemleri, Polimeraz Zincir Reaksiyonu (PCR) ve Denatüre Edici Gradient Jel Elektroforezi (DGGE) metodu uygulandı. PCR-DGGE metodu ile orşitis semptomu gösteren koçların birinden *M. bovigenitalium* ve diğerinden *M. arginini* identifiye edilirken; semptom göstermeyen bir tekeden ise *M. bovigenitalium* identifiye edildi. Elde edilen sonuçlar ile Brusellozis negatif olan koç ve tekelerin testislerinde, fertilite azalmasına yol açan orşitise neden olan Mikoplazma türlerinin varlığı ortaya konmuştur.

Anahtar sözcükler: *Genital Mikoplazmalar, PCR-DGGE, Prepusyal svap, Küçük ruminant*

INTRODUCTION

The reproductive disorders in caprines and ovines may be caused by some Mollicutes and *Mycoplasma* species [1]. *Mycoplasma* species, which are usually host specific, can infect man or animals causing clinical signs that include pneumonia, arthritis, infertility and abortion [2]. Some Mycoplasma diseases that affect small ruminants are listed

İletişim (Correspondence)
+90 392 6751000/3151
huban.gocmen@neu.edu.tr

by the OIE as they can cause major economic losses. These include contagious caprine pleuropneumonia, caused by *Mycoplasma capricolum* subsp. *capripneumoniae* in goats and contagious agalactia (CA) in sheep and goats, caused by *Mycoplasma agalactiae*, *Mycoplasma mycoides* subsp. *capri, Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma putrefaciens.* These CA infections have been associated with mastitis, arthritis, keratoconjunctivitis, abortion and atypical symptoms include respiratory and genital signs [3]. Other *Mycoplasma* species that infect small ruminants includes *Mycoplasma ovipneumoniae*, which is usually associated with respiratory disease; *Mycoplasma conjunctivae* that causes eye infections; and *M. arginini* which is thought to be a commensal, but may cause opportunistic infections. *Mycoplasma bovigenitalium*, previously called *Mycoplasma* ovine/caprine serogroup 11 [4], was originally isolated from infertile ewes having been introduced by infertile rams that showed abnormal sperm morphology and motility [5,6]. It has been associated with vulvovaginitis, epididymitis, orchitis, and infertility, resulting in economic losses.

The identification of different *Mycoplasma* species is technically difficult and expensive using conventional culture methods and mycoplasma species specific antiserum in growth inhibition tests [7]. Newer methods include specific polymerase chain reactions (PCR's) but these only identify one species unless they are multiplexed. More recently a microarray has been described but it is not widely used. The combined use of a PCR based on the 16S rDNA gene that will detect all *Mycoplasma* species, which can then be differentiated using denaturing gradient gel electrophoresis (DGGE) (PCR-DGGE) [8] to detect and identify the majority of *Mycoplasma* species is being used in a number of specialised laboratories. The aim of this study was to detect and identify mycoplasmas that cause reproductive infections by testing preputial swabs from bucks and rams.

MATERIAL and METHODS

Preputial swabs were collected from 17 bucks (Saanen) and 10 rams (Kivircik) kept at the Artificial Insemination (AI) Center of Uludag University in Turkey. The animals in semi-intensive system were mixed as bucks and rams and were kept together. All rams and bucks fed with a commercial concentrate diet with hay and water provided ad libitum. The animals were not vaccinated against CA and were clinically healthy, except for two rams which showed signs of orchitis in both testicles. Preputial swabs were placed into Stuart transport medium (cotton wrapped, Cultiplast, LP Italiana) and transported to the laboratory in cool containers at 4°C. All of samples were investigated for *Brucella* spp., *Mycoplasma* species and other bacterial infections.

Bacteriological Examination

All samples were inoculated into Mycoplasma Broth Base containing Mycoplasma Supplement G (Oxoid, UK), where ten-fold serial dilutions were made, and then plated onto Brucella selective agar (Oxoid, UK), Columbia blood agar with 5% sheep blood and MacConkey agar and Mycoplasma agar base containing Mycoplasma supplement G plates (Oxoid, UK). Columbia blood agar and MacConkey agar plates were incubated at 37°C for 24-48 h in an aerobic environment. In addition *Brucella* selective media were incubated 37°C in a 5-10% CO_2 atmosphere. Media were examined daily for up to seven days. All of the mycoplasma media were incubated at 37°C in a 5-10% CO_2 atmosphere and observed for three to seven days. The broth media were examined daily for signs of mycoplasma growth and the agar media were examined for mycoplasma-like colonies and typical 'fried-egg' colonies using a stereomicroscope. Suspect *Mycoplasma* colonies were tested for digitonin sensitivity and urease activity for Acholeplasmas and Ureaplasmas, respectively, using standard methods [7].

DNA Extraction and 16S rDNA PCR-DGGE

Genomic DNA was extracted directly from 1 mL of the broth cultures using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics). The 16S rDNA gene PCR and DGGE were performed according to the method described by McAuliffe et al.[8] using GC341-F (universal) and R543 (mycoplasma specific) primers. Briefly, DGGE was performed using the Ingeny PhorU 2x2 apparatus (GRI Molecular Biology, Essex, UK). Samples (20 µL) were loaded on to 10% polyacrylamide/bis (37.5: 1) gels with denaturing gradients from 30 to 60% (where 100% is 7M urea and 40% (v/v) deionized formamide) in 1X TAE electrophoresis buffer. Electrophoresis was carried out at 100V at a temperature of 60°C for 18 h. Gels were stained with SYBR Gold (Cambridge BioScience, UK) in 1X TAE for 30 min at room temperature and visualized under UV illumination. The controls used included the type strains of: *M. agalactiae* (NCTC 10123), *M. bovigenitalium* (NCTC 10122), *M. mycoides* subsp. *capri* (NCTC F30), *M. arginini* (NCTC 10129), *M. ovipneumoniae* (NCTC 10151), *M. bovis* (NCTC 10131), *M. capricolum* subsp. *capricolum* (NCTC 10137) and *Ureaplasma diversum*.

RESULTS

Bacterial growth was observed in 3 (1 buck and 2 rams) out of 27 samples (11.1%) as indicated by turbidity in the broths and 'fried egg' colonies on mycoplasma plates. No bacterial colonies were detected on Columbia blood agar and Brucella selective media *(Table 1)*. Digitonin sensitivity and urease activity tests were negative.

Three mycoplasma isolates were identified using the 16S rDNA PCR-DGGE method and compared to standard ruminant mycoplasma controls *(Fig. 1)*. Bacteriological examination and the 16S rRNA gene-based PCR-DGGE method identified two of the cultures as *M. bovigenitalium* and one as *M. arginini*. One of the *M. bovigenitalium* cultures and the *M. arginini* were isolated from cases of

Table 1. *Bacteriological and molecular findings obtained from preputial swabs from bucks and rams*

Host	No. of Sample	Number of *Mycoplasma* Species (positive samples) (%)	16S rDNA /DGGE Results (number of isolates)
Ram	10	2 (20%)	1 *(M. bovigenitalium)* 1 *(M. arginini)*
Buck	17	1 (5.9%)	1 *(M. bovigenitalium)*
Total	27	3 (11.1%)	3

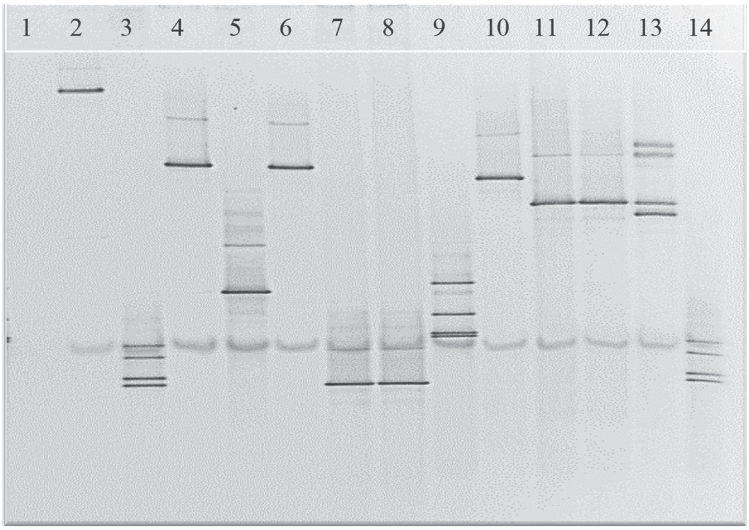


Fig 1. DGGE example gel of the amplified V3 region of 16 S rDNA gene. Lanes: 1, negative control; 2, *U. diversum* positive control; 3, *M. arginini* positive control; 4, *M. bovigenitalium* positive control; 5, *M.bovis* positive control; 6, *M.bovigenitalium* from example sample ; 7-8, *M. arginini* from example sample; 9, *M.agalactiae* positive control; 10, *M.ovipneumoniae* positive control; 11, *M.mycoides* subsp *capri* positive control; 12, unknown profile; 13, *M.capricolum* subsp *capricolum* positive control; 14, *M.arginini* 2nd positive control

orchitis in two rams and the other *M. bovigenitalium* was isolated from a buck with no apparent clinical signs.

DISCUSSION

This study confirmed of the presence of *M. bovigenitalium* and *M. arginini* in the prepuce of bucks and rams by bacteriological isolation and molecular identification. *M. bovigenitalium*, formerly known as *Mycoplasma* ovine/caprine serogroup 11, has previously been associated with abnormal sperm morphology and motility resulting in infertility in rams [1]. As its name suggests *M. bovigenitalium* has been described in cattle and it has been isolated from the reproductive tract, pneumonic, arthritic and mastitic cattle as well as aborted foetuses [9]. Lysnyansky et al.[10] identified *M. bovigenitalium* and *Mycoplasma canadense* from outbreaks of granulopapular vulvovaginitis in dairy cattle in Israel and Catania et al.[6] reported *M. bovigenitalium* from infertile cattle. There are also reports describing the isolation of *M. bovigenitalium* and *M. arginini* from the genital tract of goats [8] and semen of naturally infected asymptomatic rams [11].

Mycoplasma arginini has been isolated from a wide range of domestic animals but more commonly from sheep and goats. Rosendal [12] described it as occurring naturally in the genital tract of small ruminants. In recent years, *M. arginini* has been associated with various clinical signs in sheep and goats including pneumonia [13]. It is often thought to be a commensal or opportunistic organism and it is unusual for a mycoplasma species, in that it has been reported as occurring in many hosts, often with severe outcomes. A fatal infection due to *M. arginini* has been reported in an abattoir worker [14]. In a survey of the primary infectious agents associated with ovine ulcerative balanoposthitis and vulvovaginitis in South Africa. Kidanemariem et al.[15] identified *M. bovigenitalium* and *M. arginini* more frequently in diseased animals than healthy animals. However, Kalshingi et al.[16] isolated 34 strains of *Mycoplasma* species including *Mycoplasma bovigenitalium* and *M.arginini* from the genital tract of clinically healthy Dorper sheep and sheep with ulcerative vulvitis and balanitis.

In this study, following the bacteriological culture of *Mycoplasma* species, the PCR-DGGE method identified two *M. bovigenitalium* and one *M. arginini* from the 27 samples. Although mycoplasma culture is the standard method used by many laboratories, its success is very dependent on the laboratory receiving freshly taken samples, that have been taken aseptically, otherwise the mycoplasmas may die during transport or become overgrown by other less fastidious bacteria. This study was limited to detecting viable *Mycoplasma* species through the initial culture method. Ideally, the clinical samples should be tested immediately by PCR-DGGE, and following culture, or culture enrichment stages. The use of PCR directly on the clinical sample or from washing of the clinical sample would allow for detection of both viable and non-viable *Mycoplasma* species. Use of culture enrichment also has

the potential to further enhance the sensitivity of the PCR-DGGE method [13].

In conclusion, this study has carried out diagnostic tests for mycoplasma species that could cause infections affecting the reproductive system in male sheep and goats. It is the first report that describes the detection of *Mycoplasma* species in preputial swabs from naturally infected bucks and rams in Turkey. These results show that *Mycoplasma* species are present in the testicles of rams and bucks and were the most likely causative organisms of orchitis in the rams, which can lead to reduced fertility. In addition there is a risk that *M. bovigenitalium* can be transferred to ewes or female goats via venereal transmission. Therefore monitoring for *Mycoplasma* species by bacteriological and molecular tests such as PCR-DGGE tests are recommended for studying reproductive disorders in small ruminants. These tests can also be useful in monitoring programmes for preventing the introduction of mycoplasmas onto farms. This study highlights the usefulness of PCR-DGGE for the routine examination of ruminant samples for genital mycoplasma detection.

Bioethics and Biossecurity Committee Approval

The Scientific Ethical Committee (Uludag University. Bursa, Turkey, No: 03/07)

Declaration of Conflicting Interests

We have no conflict of interest to declare.

Acknowledgements

The authors would like to thank Roger D. Ayling, PhD., for his valuable and constructive suggestions during and development of this research work.

REFERENCES

1. Gregory L, Rizzo H, Gaeta NC, Tortorelli G, Cardroso MV, Mettifogo E, Buzinhani M, Timenetsk J: Interference of *Mycoplasma* spp. or *Ureaplasma* spp. in ovine semen quality. *J Microbiol Res,* 2 (5): 118-122, 2012. DOI: 10.5923/j.microbiology.20120205.01

2. Razin S, Yogev D, Naot Y: Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev*, 62 (4): 1094-1156, 1998.

3. Office International des Epizootes (OIE): Contagious agalactia. **In,** Terrestrial Manual. 1430-1440, 2018. http://www.oie.int/en/animal-health-in-the-world/the-world-animal-health-information-system/the-oie-data-system/; *Accessed*: 25.06.2019

4. Nicholas RAJ, Khan LA, Houshaymi B, Miles RJ, Ayling RD, Hotzel H, Sachse K: Close genetic and phenotypic relatedness between *Mycoplasma* ovine/caprine serogroup 11 and *Mycoplasma bovigenitalium*. *Syst Appl Microbiol*, 25 (3): 396-402, 2002. DOI: 10.1078/0723-2020-00121

5. Chima JC, Ojo MO, Molokwu JU, Okewole PA: Characterisation of mycoplasmas isolated from genital tract infections of sheep in Nigeria. *Rev Sci Tech*, 14 (3): 865-871, 1995.

6. Catania S, Tavella A, Gobbo F, Nicholas RAJ: Isolation of *Mycoplasma bovigenitalium* from infertile dairy cattle. *Vet Rec Case Rep,* 2:e000055, 2014. DOI: 10.1136/vetreccr-2014-000055

7. Poveda J: Biochemical characteristics in mycoplasma ıdentification. **In,** Miles R, Nicholas R (Eds): Mycoplasma Protocols. 37-50, 69-78, Humana Press Inc, New Jersey, Totowa, 1998.

8. McAuliffe L, Ellis RJ, Lawes JR, Ayling RD, Nicholas RAJ: 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. *J Med Microbiol*, 54 (8): 731-739, 2005. DOI: 10.1099/jmm.0.46058-0

9. Ruhnke H: Mycoplasmas associated with bovine genital infections. **In,** Whitford H, Rosenbusch R, Lauerman L (Eds): Mycoplasmosis in Animals. 55-61, Iowa State University Press, Ames, USA, 1994.

10. Lysnyansky I, Brenner J, Alpert N, Benjamin A, Bernstein M, Elad D, Blum S, Friedgut O, Rotenberg D: Identification of *Mycoplasma bovigenitalium* and *Mycoplasma canadense* from outbreaks of granulo-papular vulvovaginitis in dairy cattle in Israel. *Vet Rec,* 165 (11): 319-322, 2009. DOI: 10.1136/vr.165.11.319

11. Prats-Van Der Ham M, Tatay-Dualde J, de La Fe C, Paterna A, Sánchez A, Corrales JC, Contreras A, Gómez-Martín Á: Presence of *Mycoplasma agalactiae* in semen of naturally infected asymptomatic rams. *Theriogenology*, 86 (3): 791-794, 2016. DOI: 10.1016/j.theriogenology.2016.02.033

12. Rosendal S: Ovine and caprine mycoplasmas. **In,** Whitford H, Rosenbusch R, Lauerman L (Eds): Mycoplasmas in Animals: Laboratory Diagnosis. 84-92, Iowa State University Press, Ames, USA, 1994.

13. Göçmen H, Ülgen M, Çarlı KT, Önat K, Kahya S, Özdemir Ü, Mat B: Investigation of contagious agalactia by bacteriological and PCR methods in sheep and goats. *Kafkas Univ Vet Fak Derg* 21 (1): 75-80, 2015. DOI: 10.9775/kvfd.2014.11790

14. Yechouron A, Lefebvre J, Robson HG, Rose DL, Tully JG: Fatal septicemia due to *Mycoplasma arginini*: A new human zoonosis. *Clin Infect Dis,* 15 (3): 434-438, 1992.

15. Kidanemariam A, Gouws J, van Vuuren M, Gummow B: Ulcerative balanitis and vulvitis of Dorper sheep in South Africa: A study on its aetiology and clinical features. *J S Afr Vet Assoc,* 76 (4): 197-203, 2005. DOI: 10.4102/jsava.v76i4.426

16. Kalshingi HA, Bosman AM,Gouws J, van Vuuren M: Molecular characterisation of *Mycoplasma* species isolated from the genital tract of Dorper sheep in South Africa. *J S Afr Vet Assoc,* 86 (1): 1-11, 2015. DOI: 10.4102/jsava.v86i1.1199

Kafkas Univ Vet Fak Derg
26 (2): 309-312, 2020
DOI: 10.9775/kvfd.2019.22836

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Case Report

Treatment of a Full-Thickness Skin Wound in Gluteal Region of the Vervet Monkey with Topical Insulin

Çağrı GÜLTEKİN [1,a] Gökhan ULUKAN [1,b] Gökay YEŞİLOVALI [1,c]
Süleyman ÖZDEMİR [1,d] Mehmet Ege İNCE [2,e] F. Eser ÖZGENCİL [1,f]

[1] Department of Surgery, Faculty of Veterinary Medicine, Near East University, 99138, Lefkoşa, TRNC
[2] Department of Internal Medicine, Faculty of Veterinary Medicine, Near East University, 99138, Lefkoşa, TRNC
[a] ORCID: 0000-0001-8586-1472; [b] ORCID: 0000-0002-0888-6753; [c] ORCID: 0000-0002-0661-5095; [d] ORCID: 0000-0002-6146-9609;
[e] ORCID: 0000-0002-1859-1449; [f] ORCID: 0000-0003-0135-1980

Article ID: KVFD-2019-22836 Received: 14.06.2019 Accepted: 25.10.2019 Published Online: 25.10.2019

How to Cite This Article

Gültekin Ç, Ulukan G, Yeşilovalı G, Özdemir S, İnce ME, Eser Özgencil FE: Treatment of a full-thickness skin wound in gluteal region of the vervet monkey with topical insulin. *Kafkas Univ Vet Fak Derg,* 26 (2): 309-312, 2020. DOI: 10.9775/kvfd.2019.22836

Abstract

In a female vervet monkey brought to our hospital with the complaint of anaphylactic shock depending on snake bite, a gangrenous wound was detected in right gluteal region and traditional local cicatrizing and epithelizing pomade application and systemic treatment were performed for 10 days. As no signs of epithelization and shrinkiage was observed during this treatment, a new treatment protocol was started with topical insulin pomade. It was determined that epithelization started on the 4th days and completely recovered on the 46th days of the treatment and 30 days after the completion of topical insulin treatment the wound region was became hairy. It was concluded that topical treatment with insulin pomade had positive effects on delayed secondary wound healing in vervet monkeys whom their cutaneous trunci muscle *(M. panniculus carnosus)* responsible for wound contraction was not functional.

Keywords: *Insulin treatment, Secondary wound healing, Vervet monkey*

Bir Vervet Maymununun Gluteal Bölgesindeki Maddi Kayıplı Deri Yarasının Topikal İnsülin İle Sağaltımı

Öz

Hastanemize yılan ısırığına bağlı anaflaktik şok şikayeti ile getirilen dişi vervet maymununda, sağ gluteal bölgede gangrenli deri yarası tespit edildikten sonra 10 gün boyunca geleneksel lokal sikatrizan ve epitelizan pomat uygulamaları ve sistemik sağaltım yapıldı. Yarada epitelizasyon ve küçülmenin hiç gözlenmemesi üzerine topikal insulin pomat uygulamasına geçildi. Uygulamanın 4. gününde epitelizasyonun başladığı, 46. günde tamamlandığı, topikal insulin sağaltımının kesilmesinden sonra 30. günde ise epitelizasyon bölgesinin tüylendiği gözlendi. Sonuç olarak, yaranın kontraksiyonla küçülmesini sağlayan cutaneous trunci *(M. panniculus carnosus)* kasının non-human primatlarda fonksiyonel olmadığı, topikal insülin pomat uygulamalarının gecikmiş sekunder yara iyileşmesinde pozitif katkıları olduğu gözlendi.

Anahtar sözcükler: *Sekunder yara iyileşmesi, İnsülin sağaltımı, Vervet maymun*

INTRODUCTION

Wound is defined as the breakdown or loss of cellular or anatomical integrity of tissue. Healing of wound is a complex and dynamic process that combines blood vessel and cells, parenchymal cells and mediator functions and extracellular matrix production. Healing stages are classified as inflammation phase, proliferation phase, maturation and remodeling phase. Proliferation phase consists of neovascularization/angiogenesis, fibroplasia-collagen, epithelization and wound contraction phases [1]. The key point in the proliferation phase is angiogenesis, which is the formation of new blood vessels in the wound tissue. In cats and dogs, it is widely accepted that subcutaneous panniculus carnosus muscle plays an important role in providing and maintaining the cutaneous circulation, and providing wound contraction [1,2]. But the existence and development of the panniculus carnosus (cutaneous trunci) muscle in higher primates is variable and is rarely occur in the Old and New World Monkeys and completely

İletişim (Correspondence)
+90 392 6751000/3143
eserozgencil@yahoo.com

absent in human [3]. Wounds that caused by excessive tissue loss can be treated with secondary healing. Sometimes secondary healing impaired at one or more points from the stages in the normal healing process, and may lead to a delay in healing beyond the expected time [4]. Insulin is a peptide hormone and regulate blood glucose level. On the other hand, some studies show that insulin application accelerates wound healing, such as facilitates monocytes/macrophages chemotaxis, pinocytosis/phagocytosis, anabolic protein metabolism, local glucose level and secretion of inflammatory and growth mediators [5-7]. In this case report, we aimed to present the positive effects of topical insulin in the treatment of a non-healed skin wound.

CASE HISTORY

A female vervet monkey was brought to Animal Teaching Hospital, Faculty of Veterinary Medicine, Near East University with the complaint of respiratory depression and unconsciousness. In emergency examination, slow breathing, tachycardia, matt voice in the caudal lung lobes and the sternal area was determined, and was given 100% oxygen for respiratory depression and slow infusion IV colloid for 9% dehydration. Aminofilin (Filinsel® 24 mg/10 mL vial, Osel İlaç A.Ş.) was given 6 mg/kg IV. After 15 min, cardiopulmonary resuscitation was performed due to respiratory and cardiac arrest and for this aim adrenaline (Adrenalin® 1 mg/1 mL vial, Osel İlaç A.Ş.) were administered 0.5 mg IV. Based on the clinical findings and considering the development of hemothorax, thoracosynthesis was performed. Patient's respiration and heart rhythms returned to normal after 9 mL of blood was removed in the thoracosynthesis. Cephazolin (Equzolin® 1 g, 4 mL vial, Tüm Ekip İlaç A.Ş.) and enrofloxacin (10% Dufafloxacin® 100 mL vial, Dutch Farm) were administered IV against the risk of sepsis and secondary infections, and lactated Ringer's, 5% dextrose and 6% hydroxyethyl starch solution were given to increase circulation. The patient's consciousness came back after 3 h. Antibiotic use was continued for 5 days. Patient was physiologically return to normal after 1 week, but thereafter, the gangrenous area 5x5 cm in size was thought to be due to snake bite was determined in the right gluteal region *(Fig. 1a)*. Local ethacridine lactate monohydrate (Rivanol®, 1 g, Oro İlaç A.Ş.) wet dressing afterward 0.12% chlorhexidine gluconate (Klorben® spray, Drogsan San. ve Tic. A.Ş.) was used two times a day for the first 4 days with the aim of demarcation. Procaine penicillin G and potassium penicillin G (Devapen® 800 IM vial, Deva A.Ş.) were given IM for two times per day for one month for the purpose of protection against the infection as recommended by some literatures. On the 4th day of treatment, the necrotic tissues were removed by surgical debridement. After the debridement, in the wound treatment was initially used topical mixture of nitrofurazone pomade (0.2% Furacin® Pomade, Zentiva Sağlık Ürünleri Sanayi ve Tic. A.Ş), dexpanthenol pomade (Pantenol® 5% cream, Saba İlaç Sanayi ve Ticaret A.Ş), zinc oxide pomade (20% Oro® Çinko Krem, Oro İlaç A.Ş.) and centella asiatica (1% Madecassol® pomade, Bayer) two times in a day and IM administration of 5% diluted dexpanthenol (Bepanthen® 500 mg/2 mL ampoule, Bayer) once a day for 10 days *(Fig.1b)*.

In this point, we were not observed any changes in wound size and the epithelialization despite application of the mixture of 4 pomades, therefore the treatment was changed into only topical insulin pomade. The pomade mixture was prepared to contain 1 mL/100 IU of regular insulin (Humilin® R, 100 IU/mL, 3 mL cartridges, Lilly İlaç Tic. Ltd. Şti.) per 10 g of vaseline. The mixture was stored at room temperature as recommended by the insulin manufacturer and was sufficiently applied to the wound twice daily. In 5th day of topical insulin application, epithelization was observed to start. Treatment was terminated with the completion of wound epithelization on the 46th day *(Fig. 2)*. On the 30th day after the end of the treatment, it was seen that the entire region was covered with hair, except for the approximately 0.1 cm^2 area in the center of the wound *(Fig. 3)*.

DISCUSSION

Panniculus carnosus muscle plays important role in contraction and vascularization phases in wound healing in cats and dogs and some mammals [1,8,9]. But in non-human primates, panniculus carnosus muscle is primarily a shoulder muscle and arises from the superficial fascia of the lateral torso and inserts into the humerus with the mm pectoralis. Contraction of this muscle usually moves the

Fig 1. 5x5 cm in size gangrenous wound in the right gluteal region (a), despite the epithelizing and cicatrizing pomade application for 10 days after debridement, view of atonic wound (b)

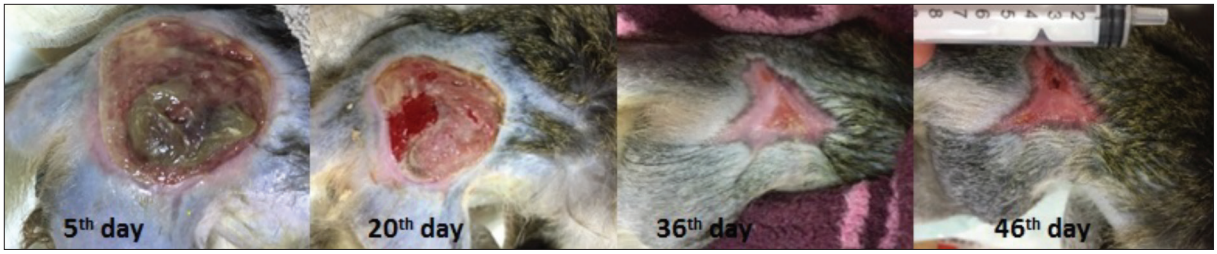


Fig 2. Appearance of the wound on different days of topical insulin treatment

Fig 3. Appearance of the gluteal region on the 30th day following topical insulin therapy was ended

skin of the side [3,10]. In our case, very slow contraction phase was observed in the right gluteal wound after one month of treatment. It was thought that slow contraction phase in the wound may be related to the absence of panniculus carnosus muscle in gluteal area in vervet monkeys.

Wounds that do not heal beyond the expected normal healing process are defined as chronic wounds [4]. Chronic wounds generally originats from an underlying problem, such as vascular failure, diabetic or pressure ulcers, and shows secondary signs such as delayed healing, discolored granulation tissue, pocketing of wound base, and wound breakdown [11]. Biofilm formation in the chronic wounds is one of the causes of delayed wound healing. A planktonic bacteria or part of the biofilm, when bound an appropriate surface, creates a biofilm and has a different structure that is durable to antimicrobials and the host's immune response. Biofilms can affect keratinocytes, fibroblasts maturation, expression of pro-inflammatory cytokines, matrix metalloproteases, and induction of angiogenesis. Furthermore, biofilm bacteria do not cause acute phase inflammation, and may not be detected using conventional sampling techniques. The biofilm treatment is more difficult and different than the normal wound treatment, therefore, it is recommended that a combination of several methods, such as systemic and local antibiotic or antiseptics, or alternative methods be used. Although the effects of systemic and topical antibiotics are limited, they are the best option for biofilm and normal wound healing [12]. The dexpanthenol is an epithelial agent commonly used in wound treatments that stimulates mRNA synthesis, fibroblastic activity and stimulates cytokines such as IL-6 and IL-8 [13]. Similarly, centella asiatica has been reported to induce angiogenesis through increased monocyte chemo-attractant protein-1 (MCP-1) expression and stimulation of Vascular endothelial growth factor expression (VEGF-1) [14]. In our case, although we use a mixture of 4 pomades that it was mentioned their effects, epithelization and wound size changes were not observed on the 10th day after debridement. We thought that the delay in wound healing was due to the suppression of the host's immune response by biofilm formation.

Diabetes mellitus (DM) is a progressive disease characterized by insulin deficiency and insulin resistance or both, and generally the insulin is used in the treatment of DM [15]. The wound healing disorder in diabetes can be attributed to various factors, such as weak blood supply, reduced proliferative potential and low inflammatory changes [16]. Some burn, surgical incision or diabetic wound animal studies were demonstrated that inadequate or non-healing wounds can be treated with topical insulin application [17-21]. In some studies, it has been observed that insulin changes the catabolic metabolism of proteins by providing positive effect on nitrogen uptake, amino acid uptake of cells, protein and DNA synthesis in local wound healing. It was also observed that insulin regulates the use of glucose in cells, reduces exudate, bacterial growth, hypoxia at the wound site, and increases vascularization, proliferation and contraction [5]. Insulin is known to provide wound healing through certain growth factors such as Alpha-smooth muscle actin (α-SMA), Insulin-like growth factor (IGF-1), Transforming Growth Factor-β1 [5,21]. It has also been reported that insulin provides endothelial cell migration without the use of the VEGF/VEGFR pathway [5]. In our case, despite the use of standard epithelization treatment and noobserved infection, we found that there was not enough epithelization in the wound. It was observed that epithelization started and accelerated after the use of insulin pomade. In our case, we thought that insulin pomade initiates proliferation and epithelialization by regulating glucose level, anabolic protein metabolism and DNA synthesis in the wound, using different growth hormone pathways and removing possible biofilm formation, in

contrast to the effects of other pomades. In conclusion, as in some mammalian studies, we think that topical insulin as an option can be used in non-healing wounds of monkeys.

REFERENCES

1. Pavletic MM: Basic principles of wound healing. **In,** Pavletic MM (Ed): Atlas of Small Animal Wound Management and Reconstructive Surgery. 4th ed., 18-34, John Wiley & Sons, USA, 2018.

2. Waldron DR, Zimmerman-pope N: Skin and adnexia. **In,** Slatter D (Ed): Textbook of Small Animal Surgery. 3rd ed., 259-274, WB Saunders, USA, 2003.

3. Turnquist JE, Nancy Minugh-Purvis N: Functional morphology. **In**, Abee CR, Mansfield K, Tardif SD, Morris T (Eds): Nonhuman Primates in Biomedical Research: Biology and Management. 2nd ed., 87-129, Elsevier, USA, 2012.

4. Enoch S, Leaper DJ: Basic science of wound healing. *Surgery*, 23 (2): 37-42, 2005. DOI: 10.1383/surg.23.2.37.60352

5. Hrynyk M, Neufeld RJ: Insulin and wound healing. *Burns*, 40 (8): 1433-1446, 2014. DOI: 10.1016/j.burns.2014.03.020

6. Chen X, Liu Y, Zhang X: Topical insulin application improves healing by regulating the wound inflammatory response. *Wound Repair Regen*, 20 (3): 425-434, 2012. DOI: 10.1111/j.1524-475X.2012.00792.x

7. Abdelkader DH, Osman MA, Elgizaway SA, Faheem A, McCarron PA: The role of insulin in wound healing process: Mechanism of action and pharmaceutical applications. *J Anal Pharm Res,* 2 (1): 00007, 2016. DOI: 10.15406/japlr.2016.02.00007

8. Naldaiz-Gastesi N, Bahri OA, Lopez de Munain A, McCullagh KJ, Izeta A: The *panniculus carnosus muscle*: An evolutionary enigma at the intersection of distinct research fields. *J Anat*, 233 (3): 275-288, 2018. DOI: 10.1111/joa.12840

9. Koç B, Özaydın İ, Özba B, Ünsaldı E, Okumuş Z: Doku kayıplı gangrenli açık yaralarda lokal insülin ve pinch gref uygulamaları ile iyileşmenin hızlandırılması üzerinde klinik gözlemler. *2. Ulusal Veteriner Cerrahi Kongresi,* 1-2 Ekim, s.61-65, Alata - Mersin/Türkiye, 1990.

10. Oxnard CE, Franklin D: Ghosts of the past II: Muscles and fasciae in the primate forelimb domain. *Folia Primatol*, 79 (6): 441-457, 2008. DOI: 10.1159/000151357

11. Fierheller M, Sibbald RG: A clinical investigation into the relationship between increased periwound skin temperature and local wound infection in patients with chronic leg ulcers. *Adv Skin Wound Care*, 23 (8): 369-379, 2010. DOI: 10.1097/01.ASW.0000383197.28192.98

12. Rhoads DD, Wolcott RD, Percival SL: Biofilms in wounds: Management strategies. *J Wound Care*, 17 (11): 502-508, 2008. DOI: 10.12968/jowc.2008.17.11.31479

13. Proksch E, de Bony R, Trapp S, Boudon S: Topical use of dexpanthenol: A 70th anniversary article. *J Dermatolog Treat*, 28 (8): 766-773, 2017. DOI: 10.1080/09546634.2017.1325310

14. Morgan C, Nigam Y: Naturally derived factors and their role in the promotion of angiogenesis for the healing of chronic wounds. *Angiogenesis*, 16 (3): 493-502, 2013. DOI: 10.1007/s10456-013-9341-1

15. Modi P: Diabetes beyond insulin: Review of new drugs for treatment of diabetes mellitus. *Curr Drug Discov Technol*, 4 (1): 39-47, 2007. DOI: 10.2174/157016307781115476

16. Sharma A, Singh AK, Warren J, Thangapazham RL, Maheshwari RK: Differential regulation of angiogenic genes in diabetic wound healing. *J Invest Dermatol*, 126, 2323-2331, 2006. DOI: 10.1038/sj.jid.5700428

17. Greenway SE, Filler LE, Greenway FL: Topical insulin in wound healing: A randomised, double-blind, placebo-controlled trial. *J Wound Care*, 8 (10): 526-528, 1999. DOI: 10.12968/jowc.1999.8.10.26217

18. Lima MHM, Caricilli AM, de Abreu LL, Araújo EP, Pelegrinelli FF, Thirone CPA, Tsukumo DM, Pessoa AFM, dos Santos MF, de Moraes MA, Carvalheira JBC, Velloso LA, Saad MJA: Topical insulin accelerates wound healing in diabetes by enhancing the AKT and ERK pathways: A double-blind placebo-controlled clinical trial. *PloS One,* 7 (5): e36974, 2012. DOI: 10.1371/journal.pone.0036974

19. Achar RAN, Silva TC, Achar E, Martines RB, Machado JLM: Use of insulin-like growth factor in the healing of open wounds in diabetic and non-diabetic rats. *Acta Cir Bras*, 29 (2): 125-131, 2014. DOI: 10.1590/S0102-86502014000200009

20. Azevedo F, Pessoa A, Moreira G, Dos Santos M, Liberti E, Araujo E, Carvalho C, Saad M, Lima MH: Effect of topical insulin on second-degree burns in diabetic rats. *Biol Res Nurs*, 18 (2): 181-192, 2016. DOI: 10.1177/1099800415592175

21. Özaydin İ, Aksoy Ö, Yayla S, Kurt B, Kilic E, Bingöl SA, Can I, Deprem T: Clinical, histopathological and immunohistochemical evaluation of the effects of topical NPH-insulin on full-thickness open wounds: An *in vivo* study in diabetic and non-diabetic mice. *Ankara Univ Vet Fak Derg,* 65 (3): 219-228, 2018. DOI: 10.1501/Vetfak_0000002850

INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given in the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal cowers all aspects of veterinary medicine and animal science.

In the interests of brevity and standalone readability, Kafkas Universitesi Veteriner Fakultesi Dergisi strongly discourages the submission of multi-part manuscripts. Authors who feel that their topic requires an exception should obtain approval from the editor before submission of a multi-part manuscript. If submitted, multipart papers can be assigned to different editorial board members and independent outside expert reviewers. It is necessary to load all parts of manuscript are required to be loaded into the online system at the same time.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Transfer Agreement Form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Types of Manuscripts

Original (full-length) Manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit not include tables and illustrations. Abstract should contain 200±20 words.

Short Communication Manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the abstracts should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in

total. The page limit not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case Reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow Introduction, Case History, Discussion and References. The length of the text should be no longer than 4 pages in total. The page limit not include tables and illustrations.

Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 2 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow Introduction, text (with appropriate titles), conclusion, and references. The length of the text should be no longer than 15 pages in total.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

6- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

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 be complied with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote style of Kafkas Universitesi Veteriner Fakultesi Dergisi; https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

8- The editorial board has the right to perform necessary modifications and reduction on the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

9- All responsibilities from published articles merely belong to the authors. According to ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with broad database of academic publications.

10- There is no copyright fee for the authors.

11- A fee is charged from the authors to cover printing cost and other expenses. This payment information can be found at *http://vetdergi.kafkas.edu.tr/*

12- Reprints (in multiples of 50) of the article are sent to the authors for free.

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover Letter (without author/authors name)

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction)

• Other information has been added that should be known by the editorial board (e.g.; list of other journal or conference papers (if any) published or submitted by you or any co-author)

• Authors should add the necessary clarifications about editor//adviser/reviewer's comments to the cover letter section for each revision.

All necessary files have been uploaded

- Title page

• Include title, running title (no more than 5 words)

• The author's name, institutional affiliation

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Include title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Graphical abstracts/Highlights files (where applicable)

- Supplemental files (where applicable)

Further considerations

• Journal policies detailed in this guide have been reviewed

• Manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Acknowledgement and conflicts of interest statement provided