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İÇİNDEKİLER (Contents)

ARAŞTIRMA MAKALELERİ (Research Articles)	Sayfa (Page)
Detection and Molecular Examination of Pathogens in Honey and Bees in the Northern Marmara Region, Turkey (Kuzey Marmara Bölgesindeki Bal ve Arılardaki Patojenlerin Tespiti ve Moleküler İncelenmesi) BAYRAKAL GM, GÖZDE EKİCİ G, AKKAYA H, SEZGİN FH, DÜMEN E (DOI: 10.9775/kvfd.2019.22845)	313
Immunohistochemical Distributions of HGF and PCNA in the Kidneys of Diabetic and Non-Diabetic Mice (Diyabetik ve Nondiyabetik Farelerin Böbrek Dokusunda HGF ve PCNA'nın İmmunohistokimyasal Dağılımı) DEPREM T, İLHAN AKSU S, KORAL TAŞÇI S, BİNGÖL SA, GÜLMEZ N, ASLAN Ş (DOI: 10.9775/kvfd.2019.23002)	321
Phylogenetic Grouping and Antimicrobial Resistance Profiles of *Escherichia coli* Isolated from Calves in Xinjiang, China (Çin'in Sincan Bölgesindeki Buzağılardan İzole Edilen *Escherichia coli*'nin Filogenetik Gruplandırması ve Antimikrobiyal Direnç Profili) ZHANG X, LI J, ZHANG L, QIAO J, MENG Q, CHEN Y, CAI K, HUANG X, WU T, HAN M, ZHONG F (DOI: 10.9775/kvfd.2019.23046)	329
Comparison of Culture and PCR for Detection of Field Isolates of Bovine Milk Mollicutes (Sığır Sütü Mollekütlerinin Saha İzolatlarının Tespiti İçin Kültür ve PCR Yöntemlerinin Karşılaştırması) AL-FARHA AAB, HEMMATZADEH F, TEARLE R, JOZANI R, HOARE A, PETROVSKI K (DOI: 10.9775/kvfd.2019.23106)	337
The Use of Glauconite as a Feed Additive in Broiler Nutrition and Its Effect on Growth Performance, Intestinal Histomorphology and Biomechanical Properties of Bones (Broyler Beslemede Glokonitin Yem Katkı Maddesi Olarak Kullanımı ve Büyüme Performansı, Bağırsak Histomorfolojisi ve Kemiklerin Biyomekanik Özellikleri Üzerine Etkisi) DURNA AYDIN Ö, YILDIZ G, GÜNTÜRKÜN OB, BAYRAKTAROĞLU AG (DOI: 10.9775/kvfd.2019.23154)	343
The Levels of Trace Elements and Macrominerals in Calves with Sepsis (Sepsisli Buzağılarda İz Elementler ve Makrominerallerin Seviyeleri) COSKUN A, AYDOGDU U, GUZELBEKTES H, SEN I (DOI: 10.9775/kvfd.2019.23187)	351
Screening of Chinese Medicinal Herbs for Anthelmintic Efficacy Against *Gyrodactylus kobayashii* (Monogenea) in Goldfish *(Carassius auratus)* (Japon Balıklarında *[Carassius auratus] Gyrodactylus kobayashii*'ye [Monogenea] Karşı Çin Şifalı Otlarının Anthelmintik Etkinliklerinin İncelenmesi) LIAN K, ZHANG M, ZHOU L, SONG Y, GUAN X (DOI: 10.9775/kvfd.2019.23196)	357
Higenamine Decreased Oxidative Kidney Damage Induced By Ischemia Reperfusion in Rats (Higenamin Ratlarda İskemi Reperfüzyonunun Neden Olduğu Oksidatif Böbrek Hasarını Azaltır) GÜLER MC, TANYELİ A, ERASLAN E, EKİNCİ AKDEMİR FN, NACAR T, TOPDAĞI Ö (DOI: 10.9775/kvfd.2019.23250)	365
Generation of Ectodysplasin A *(eda)*-targeted Knockout Zebrafish Via the CRISPR/Cas9 System (CRISPR/Cas9 Sistemi Kullanılarak Ectodysplasin A *[eda]* İfade Etmeyen Zebra Balığı Üretimi) ZHANG C, HU L, LIU S, WANG W, ZHAO K (DOI: 10.9775/kvfd.2019.23252)	371
Detection of BVDV 1q in China: Genetic Characterization and Experimental Infection for the Investigation of It's Pathogenicity (Çin'de BVDV 1q'nun Tespiti: Patojenitesinin Araştırılması Amacıyla Genetik Karakterizasyon ve Deneysel Enfeksiyon) HE Y, MA X, HUANG X, SHENG J, ZHONG F, ZHAO X, ZHANG Y, CHEN C (DOI: 10.9775/kvfd.2019.23273)	377
Effects of Cleavers *(Galium aparine)* and Yarrow *(Achillea millefolium)* Extracts on Rumen Microbial Fermentation in *In-vitro* Semi-Continuous Culture System (RUSITEC) (Yoğurt Otu *[Galium aparine*] ve Civan Perçemi *[Achillea millefolium]* Ekstraktlarının *In-vitro* Yarı-Sürekli Kültür Sisteminde [RUSITEC] Rumen Mikrobiyal Fermentasyonu Üzerine Etkileri) DEMIRTAS A, MUSA SAA, PEKCAN M, SALGIRLI DEMIRBAS Y, PISKIN I, EMRE B, TOPRA N, OZTURK H (DOI: 10.9775/kvfd.2019.23283)	385
A Molecular Investigation of Carbapenem Resistant Enterobacteriaceae and bla_{KPC}, bla_{NDM} and bla_{OXA-48} Genes in Raw Milk (Çiğ Sütlerde Karbapenem Dirençli Enterobacteriaceae ve bla_{KPC}, bla_{NDM} ve bla_{OXA-48} Gen Varlığının Moleküler Olarak İncelenmesi) AL S, HIZLISOY H, ERTAŞ ONMAN Z, KARADAL F, BAREL M, YILDIRIM Y, GÖNÜLALAN Z (DOI: 10.9775/kvfd.2019.23329)	391
Evaluation of Serum Amyloid A and Procalcitonin in Some Inflammatory Diseases of Cattle (Sığırların Bazı İnflamatuar Hastalıklarında Serum Amiloid A ve Prokalsitoninin Değerlendirilmesi) BASBUG O, YURDAKUL I, YUKSEL M (DOI: 10.9775/kvfd.2019.23412)	397
Expression Profiles of Toll-like Receptors 2, 7 and 8 in Rat Testis and Epididymis During Postnatal Developmental Period (Postnatal Gelişim Döneminde Rat Testis ve Epididimisinde Toll-benzeri Reseptörler 2, 7 ve 8'in Ekspresyon Profilleri) ÖZTOP M, ÖZBEK M, ERGÜN E, ERGÜN L, BEYAZ F, ERHAN F, KANDİL B (DOI: 10.9775/kvfd.2019.23436)	403
Antimicrobial Effects of *Thymus vulgaris, Cinnamomum zeylanicum* and *Zingiber officinale* Essential Oils on *Salmonella enterica* serovar Enteritidis *(Thymus vulgaris, Cinnamomum zeylanicum* ve ***Zingiber officinale*** Uçucu Yağlarının *Salmonella enterica* serovar Enteritidis'e Antimikrobiyal Etkileri) DÜLGER D, ALBUZ Ö (DOI: 10.9775/kvfd.2019.23456)	413

A Mutant of *Listeria monocytogenes* Shows Decreased Virulence and Confers Protection Against Listeriosis in Mice (Farelerde Düşük Virulans Gösteren ve Listeriosise Karşı Koruma Sağlayan *Listeria monocytogenes* Mutantı) REN, YANG M, WANG P, JIANG J, YAN G (DOI: 10.9775/kvfd.2019.23466)	419
The Effects of Calcium Aluminate and Calcium Silicate Cements Implantation on Haematological Profile in Rats (Kalsiyum Alüminat ve Kalsiyum Silikat Sement İmplantasyonunun Ratlarda Hematolojik Profil Üzerine Etkileri) KATICA M, JANKOVIĆ O, TANDIR F, GRADAŠČEVIĆ N, DEKIĆ R, MANOJLOVIĆ M, PARAŠ S, TADIĆ- LATINOVIĆ L (DOI: 10.9775/kvfd.2019.23476)	427
Effect of Different Preservation and Salting Methods on Some Volatile Compounds and Sensory Properties of Kashar Cheese (Kaşar Peynirinde Farklı Muhafaza ve Tuzlama Yöntemlerinin Bazı Uçucu Bileşenleri ve Duyusal Özellikleri Üzerine Etkisi) ÇETİNKAYA A (DOI: 10.9775/kvfd.2019.23508)	435
OLGU SUNUMU (Case Report)	
Low-field Magnetic Resonance Imaging of Changes Accompanying Slipped Capital Femoral Epiphysis in a Cat (Bir Kedide Kaymış Femur Başı Epifizine Bağlı Değişikliklerin Düşük-alan Manyetik Rezonans Görüntüleme İle Belirlenmesi) GŁODEK J, MIESZKOWSKA M, ADAMIAK Z (DOI: 10.9775/kvfd.2019.23330)	445
Abdominal Wall Hibernoma in a Cat: A Case Report (Bir Kedide Abdominal Hibernoma: Olgu Sunumu) OZTURK-GURGEN H, EGEDEN E, CALP-EGEDEN O, GUREL A (DOI: 10.9775/kvfd.2019.23504)	449
EDİTÖRE MEKTUP (Letter to the Editor)	
Two Cases of "Celox" Gauze Application to Control Bleeding from the Canine Popliteal Artery (İki Köpekte Popliteal Arter Kanamasının Kontrolü İçin "Celox" Gazlı Bez Uygulaması) OZTURK-GURGEN H, EGEDEN E, CALP-EGEDEN O, GUREL A (DOI: 10.9775/kvfd.2020.24022)	453

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Research Article

Detection and Molecular Examination of Pathogens in Honey and Bees in the Northern Marmara Region, Turkey [1]

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Abstract

Honey, which has many positive health effects, is fondly consumed in our country and in the world. Although honey is considered to be a micro-organism-free food because of its antimicrobial and bacteriostatic effects, studies refute this idea. In addition to primary contamination, personnel, tools and equipment used in beekeeping and honey production is a potential source of secondary contamination. In addition, honey, which can carry many microorganisms as a result of cross-contamination, is among the important foods and can threat public health. Therefore, it is thought that screening of pathogens that may be present in honey would contribute to the studies. Due to the geographical location, the diversity of climate and vegetation, Turkey is located in the upper row of honey producting countries. In this study, 900 samples examined in Kırklareli province in Northern Marmara Region. Kırklareli region has been chosen as the research area since it is considered as an important province in honey production and is a border province located in the industrial zone. The aim of the study was to investigate the presence of parasitological, bacterial, fungal and viral parameters which are important for the quality of the consumer, bee, larvae, colony and honey. According to the obtained data, positive results were found in many parameters and statistically significant results were obtained.

Keywords: *Honey, Bee, Larvae, Microbial quality, Pathogen*

Kuzey Marmara Bölgesindeki Bal ve Arılardaki Patojenlerin Tespiti ve Moleküler İncelenmesi

Öz

Sağlık açısından birçok olumlu etkiye sahip bal ülkemizde ve dünyada severek tüketilmektedir. Antimikrobiyal ve bakteriyostatik etkileri sebebiyle bal mikroorganizma içermeyen bir gıda olarak düşünülmesine rağmen yapılan çalışmalar bunun aksini ispatlamaktadır. Primer kontaminasyonun yanısıra, arıcılık ve bal üretiminde kullanılan araç, gereçler ve personel potansiyel bir sekonder kontaminasyon kaynağıdır. Ayrıca çapraz kontaminasyonlar sonucunda da birçok mikroorganizmanın taşıyıcılığını yapabilen bal halk sağlığını tehdit edebilme potansiyeline sahip önemli gıdalar arasında yer almaktadır. Bu nedenle ballarda bulunabilecek patojenlerin taranmasının literatüre katkı sağlayacağı ve benzer çalışmalara temel oluşturacağı düşünülmüştür. Coğrafi konumu, iklim ve bitki örtüsü çeşitliliği sebebiyle Türkiye bal üretiminde üst sıralarda yer almaktadır. Çalışmamızda, Kuzey Marmara bölgemizde bulunan Kırklareli ilimizdeki 900 adet örneği incelenerek tüketici, arı, larva, koloni ve bal kalitesi açısından önem arz eden parazitolojik, bakteriyel, fungal ve viral parametrelerin varlığının araştırılması amaçlanmıştır. Hem bal üretiminde önemli iller arasında sayılması hem de sanayi bölgesinde yer alan bir sınır ilimiz olması sebebiyle araştırma alanı olarak Kırklareli bölgesi seçilmiştir. Elde edilen veriler doğrultusunda birçok parametrede pozitif sonuçlar bulunmuş ve istatistiksel olarak değerlendirildiğinde anlamlı sonuçlar elde edilmiştir.

Anahtar sözcükler: *Bal, Arı, Larva, Mikrobiyal kalite, Patojen*

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INTRODUCTION

Turkey, which has 7 different geographical regions with its own unique climate and vegetation, is an important country for honey production. Our country also contains 75% of the honey plant species identified in the world [1,2].

Honey contains fructose (~38%), glucose (~30%), sucrose (~1-2%), other carbohydrates (~12%), various minerals (~0.2%), proteins (~200 mg/100 g), and water (~17%), and is a nutritionally important food source [3]. Honey has been used as a therapeutic agent in ancient times due to its antimicrobial effect as well as health benefits to the consumer [4,5].

In general, honey pH ranges between 3.4-6.1 and water activities range from 0.5-0.6. Osmolarity, pH and hydrogen peroxide activities are considered as important factors that induce antimicrobial effect. The basic principle for this antimicrobial activity is the oxidation of glucose through the enzyme glucose-oxidase, resulting in the appearance of hydrogen peroxide. However, the antimicrobial effect described above can only be generated by successfully diluting the honey, and hydrogen peroxide, which may be sufficient for antimicrobial activity, cannot be produced in sufficient amounts due to the low water activity of the honey under normal conditions [6-8]. Although some honey types contain some phenolic compounds based on residual non-hydrogen peroxide (such as benzoic acid and some flavonoids) and a small amount of pathogenic microorganism is expected in honey due to the compounds in question, minimum hygiene rules and risk for consumer health in honey produced without food safety systems, it is reported that pathogens may be found as a factor [9].

Foodborne pathogens are considered to be an important risk factor for public health in developed and developing countries because of their worldwide spread. In the United States alone, 76 million cases of food poisoning occur every year, 325.000 of these cases are hospitalized, and 5.000 of the hospitalized cases are reported as fatal or deadly cases [10]. Viruses, bacteria, fungi and parasitic mites are the most common disease factors in beekeeping [11]. The fecal-oral route is an important way for these diseases transmission. The agents contaminating bees through water and food can be transmitted to larvae and pupae by infected bees [12]. Another contamination that may occur in honey is seconder contamination caused by secondary sources of contamination such as personnel, tools and equipment.

Serological methods, electron microscopy (EM) and agar gel immunodiffusion (AGID) test are used for the detection of viruses. However, due to the low specificity, low sensitivity of these methods and the inability to detect latent infections, the use of molecular analysis methods has started to increase [11,13]. Although there are many studies with the antimicrobial and bacteriostatic effects of honey in medical literature, the information about the food-borne pathogens in the initial flora of honey and information about the reproductive profile of these pathogens is limited [8,14].

In this research presence of total mesophilic aerobic bacteria, total coliform bacteria, *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus)*, *Clostridium botulinum (C. botulinum)*, *Nosema* spp., *Ascosphaera apis (A. apis)*, *Aspergillus flavus (A. flavus)*, *Aspergillus fumigatus (A. fumigatus)*, *Varroa* spp. was investigated using classical methods. Molecular analysis method (Reverse Transcription-Polymerase Chain Reaction-RT-PCR) was used for investigation of Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV) and Sacbrood virus (SBV).

With this investigation, it was aimed to determine if honey bees and honey production threat to public health in Kırklareli, which is an important place for beekeeping in Turkey.

MATERIAL and METHODS

Sample Collection

The bees, honey and larvae samples collected for analysis for the determined microbiological parameters were collected from direct bee colonies according to cold chain standards and transferred to the laboratory. Visually, the hives which were stagnant, weak and showing disease symptoms (walking on flight boards by swarming their abdomen, wings discrete bees, thrown larvae, slimy-diarrhea soil crumbs, dead *Varroa* etc.) were preferred primarily in the sampling procedure. 10x10 cm² honeycomb honey samples (capping or uncapping) were taken. During the collection of adult bee (at least 150 and over from each colony) and larvae samples (10x10 cm² larvae honeycomb from each colony), the samples were collected from the newly dead and live bees. 900 samples were collected from 300 hives belonging to 300 families that provided the main livelihood sources from beekeeping from 9 districts/regions of Kırklareli province in Northern Marmara Region of Turkey.

Microbiological Analysis

Sample Preperation: 10 g of each samples were aseptically taken and homogenized with 90 mL of sterile saline water. Serial decimal dilutions were prepared from initial homogenate in the same sterile diluents.

Total Mesophilic Aerobic Bacteria: Petri dishes including Plate Count Agar (PCA) and sample diluent were incubated at 35ºC for 48 h and the counting of the typical colonies was performed at the end of the incubation period [15].

Total Coliform Bacteria: 1 mL aliquots of each sample dilution transferred to petri dishes including Violet Red Bile Agar Petri dishes were incubated at 18-24 h at 35°C and the counting of the typical colonies was performed at the end of the incubation period [16].

***Escherichia coli*:** 1 mL dilution was added to petri dishes including Tryptone Bile X-glucuronide Agar (TBX) and incubated at 44°C for 18-24 h [17]. In addition to TBX agar, a chromogenic medium containing 4-methyl-umbelliferyl-β-D-glucuronide was used for verification.

***Staphylococcus aureus*:** After 1 mL dilution was added to petri dishes including Baird Parker agar (BPA) with 5% egg yolk tellurite emulsion, petri dishes were incubated at 37°C for 24-48 h. DNase agar and coagulase test were used for confirmation [18].

***Clostridium botulinum*:** Samples were inoculated into cooked meat medium (CMM) and Trypticase-peptone-glucose-yeast extract (TPGY) for the enrichment of cultures. After incubation time (5-10 days), cultures were streaked to anaerobic Egg Yolk Agar and incubated at 35°C for about 48 h under anaerobic conditions. For the honey samples isolation, dilution centrifugation and supernatant filtration methods were used and then isolation samples were added to CMM and TPGY [19].

***Ascosphaera apis*:** Samples were cultured on potato dextrose agar (PDA) at 30°C for 5-8 days. After incubation suspected colonies were examined [20].

***Aspergillus flavus* and *Aspergillus fumigatus*:** Samples were spread on Di-Chloran Rose Bengal Medium, Czapek's Dox Agar Medium and Potato Dextrose Medium and incubated at 25-30°C [21,22].

***Nosema* spp.:** Intestinal specimens of up to 30 adult bees from each colony were homogenized after extraction. After each sample were homogenized, 1 mL homogenate in 1 mLdistilled H_2O were counted in a haemocytometer (Neubauer chamber) under microscope for the presence of *Nosema* spp. spores [23,24]. After homogenization of honey samples taken from the same hive, approximately 1 mL honey sample was taken and 1 mL distilled water was added to per sample. Homogenates were placed on Neubauer slide and microscopic examination was performed.

***Varroa* spp.:** In order to demonstrate the presence of *Varroa* to beekeepers in the field practically, powder sugar shake method was used for detection of *Varroa* mites [25]. Bees and powder sugar were placed into a jar and the jar was shekan. After the mixture in the jar was poured onto a white cover, *Varroa* mites were counted. In the laboratory examination, adult bees were put into a bottle, shaken with gasoline and filtered through a double honey strainer. The *Varroas* shaken on white blotter were counted. The probable *Varroas* of bees were also detected by stereo-microscopic analysis. For larval analysis, larvae and honeycomb cells were frozen. After freezing, it was disintegrated and filtered through a double honey strainer. Finally, it was counted after poured it on white blotter paper.

Table 1. *RT-PCR primers used for viruses selected in the study*

Primer Name	Primer Sequence	Product Size (Bp)
ABPV 1	5'-agccactatgtgctatcgtat-3'	207
ABPV 2	5'-atggtgacctctgtgtcatta-3'	207
BQCV 3	5'-gcaagctcttccaatgatag-3'	322
BQCV 4	5'-aagattcagccgagtcctta-3'	322
SBV 5	5'-accaaccgattcctcagtag-3'	487
SBV 6	5'-ccttggaactctgctgtgta-3'	487

Molecular Analysis

In our study, the collected samples were investigated for the honey bee viruses. ABPV, BQCV and SBV were analyzed. For the negative control samples, honey samples obtained from the hives belonging to Istanbul University Faculty of Veterinary, Parasitology Department were used. For this purpose, honey bee/honeycomb/pupa samples collected from different hives (preferably dead) were recorded according to the hives from which they were collected and the samples collected from each hive were homogenized separately on the hive basis, but together on the basis of the sample. Viral RNA contents were extracted from homogenates using the Garbensteiner method using purification and extraction kits [26]. The specific primer sets (ABPV; GenBank Accession No. NC_002548, BQCV; GenBank Accession No. AF183905, SBV; GenBank Accession No. NC_002066), which have been previously issued and approved by the reference laboratories, are provided to be commercially designed [13]. The primary sets used in our study are shown in *Table 1*.

Statistical Analysis

The Kendall's tau b correlation coefficient can be used to test whether two variables are statistically interdependent. Values of Tau-b range from -1 to +1 (100% positive association, or perfect agreement). Kendall's tau b correlation coefficient was used to compare the correlations between each of the study variables [27].

RESULTS

In this study, 900 samples were collected from 300 hives. The presence of parasitological, bacterial and viral parameters of the samples which are important for the quality of, bees, larvae, colony and honey were investigated. Samples collected from bees, honeycomb honey and larvae (bee, honey and larvae samples from each hive) were examined in terms of 13 different parameters, 5 bacterial, 3 fungal, 2 parasitological and 3 viral parameters. The results obtained from the study are shown in the tables *(Table 2, 3, 4)* below.

The numbers mentioned in the sections in the table are the number of samples belonging to the related parameter, which are considered as risky for bee and hive health.

Table 2. *The results of bee samples (n = 300 colonies) in terms of examined parameters*

Parameter	District								
	Merkez (n=30)	Ulukonak (n=35)	Çağlayık (n=38)	Lüleburgaz (n=41)	Kofcaz (n=29)	Demirköy (n=37)	Pehlivanköy (n=22)	Vize (n=31)	Babaeski (n=37)
Staphylococcus aureus	4	8	9	15	0	11	12	14	17
Clostridium botulinum	0	5	3	4	0	0	6	4	0
Ascosphaera apis	2	3	1	5	0	6	4	2	7
Aspergillus flavus	4	0	7	11	0	2	6	1	12
Aspergillus fumigatus	4	0	7	11	0	2	6	1	12
Nosema spp.	6	10	5	7	2	7	5	3	11
Varroa spp.	8	4	10	2	3	4	8	1	4
ABPV	0	0	0	0	0	0	0	0	0
BQCV	0	0	0	0	0	0	0	0	0
SBV	0	0	0	0	0	0	0	0	0

Table 3. *The results of larvae samples (n = 300 colonies) in terms of examined parameters*

Parameter	District								
	Merkez (n=30)	Ulukonak (n=35)	Çağlayık (n=38)	Lüleburgaz (n=41)	Kofcaz (n=29)	Demirköy (n=37)	Pehlivanköy (n=22)	Vize (n=31)	Babaeski (n=37)
Staphylococcus aureus	6	7	11	8	0	13	8	12	19
Clostridium botulinum	0	3	4	6	1	2	3	1	0
Ascosphaera apis	4	2	1	2	0	3	5	0	11
Aspergillus flavus	2	0	5	8	0	0	3	1	6
Aspergillus fumigatus	2	0	5	8	0	0	3	1	6
Varroa spp.	10	6	7	6	5	6	7	3	6
ABPV	0	0	0	0	0	0	0	0	0
BQCV	0	0	0	0	0	0	0	0	0
SBV	0	0	0	0	0	0	0	0	0

Table 4. *The results of honey samples (n = 300) in terms of examined parameters*

Parameter	District								
	Merkez (n=30)	Ulukonak (n=35)	Çağlayık (n=38)	Lüleburgaz (n=41)	Kofcaz (n=29)	Demirköy (n=37)	Pehlivanköy (n=22)	Vize (n=31)	Babaeski (n=37)
Total Mesophilic Aerobic Bacteria	12	9	11	10	6	2	8	9	7
Total Coliform Bacteria	7	12	15	11	4	8	13	2	12
Escherichia coli	3	5	4	8	0	0	2	0	5
Staphylococcus aureus	8	9	3	0	0	4	3	19	11
Clostridium botulinum	0	2	3	0	0	0	2	3	0
Ascosphaera apis	2	0	1	2	2	0	0	1	3
Aspergillus flavus	2	1	3	9	0	5	7	3	9
Aspergillus fumigatus	2	1	3	9	0	5	7	3	9
Nosema spp.	0	0	0	0	0	0	0	0	0
Varroa spp.	0	0	0	0	0	0	0	0	0
ABPV	0	0	0	0	0	0	0	0	0
BQCV	0	0	0	0	0	0	0	0	0
SBV	0	0	0	0	0	0	0	0	0

Samples that were positive for the analyzed pathogens (in a single colony - diffuse appearance range) were evaluated as risky for bee, honey, larvae and consumer health.

In some beehives, although the agents were isolated, no significant symptoms related to the diseases caused by that agent were observed. It was concluded that all beehive bees could be at risk by evaluating the possibility that the disease may have in hives that do not show symptoms but contain agents.

All the correlations of the bees, larvae and honeycomb honey samples were examined in terms of the microbiological parameters analyzed. Since the viral agents (acute bee paralysis virus, black queen cell virus and Sacbrood virus) were not detected in any of the samples, the parameters were excluded.

DISCUSSION

Honey is a very optimal nutrient for people of all ages, except for the first year after birth [28]. In particular, the nutrients in their contents ensure that both the nutritional value of honey is very high and helps to activate the human immune system against many diseases. Best quality honey in the world is produced in Turkey by reason of having many flower species. Although honey production is incrising in Turkey, it is below the required level in terms of honey export. The most important reason for this imbalance between production and exports is the parameters of microbiologic origin which are transmitted to the hives from primary/secondary contamination sources. The fact that most of the bacterial, fungal and parasitic factors analyzed in our study were positive proved that these factors can survive in honey. This contamination indicates that hygienic criteria are not sufficient for honey production.

In addition to honey, bees are also affected by many factors and they are infected with pathogenic, viral and parasitic factors [29]. Pathogens can be transmitted to bees and larvaes via other bees, parasitic mite or environmental factors such as beekeeping equipments and air [30]. While the pathogens of bees and larvae causes economic damage, at the same time, they can pose a risk to public health as a result of contamination of honey by disease factors. In our study, when the honey and larvae were examined, the presence of many prameters was detected, and only the viral parameter were not positive. In terms of the examined hives, there is a risk that many bees may get sick or carry these factors both to the larvae and other bees.

When the bacterial analysis results obtained in our study are examined, honey samples which are considered as the highest number of risks for consumer health in terms of total mesophilic aerobic bacteria parameters are collected from Kırklareli Center, Çağlayık and Lüleburgaz regions. The main reason for this situation is the location features of the study region. Pollen, dust, air, industrialization, incorrect waste management, the digestive tracts of honey bees and flowers are the main source and cause of contamination [31,32]. There is more population, vehicle traffic and waste potential in Kırklareli central region. There is more circulation of people/animals/vehicles/goods on the borders due to the fact that Çağlayık region is on the border with Bulgaria, and Lüleburgaz region is the industrial zone of Kırklareli province. Tysett et al.[33] reported in a study that they isolated species belonging to the family *Bacillus*, *Enterobacter* and *Micrococcus* in all honey samples examined. In our study, as in the study of Tysett et al.[33] incidence values were determined to threaten the health of the consumer. The height of these values is attributed to the rapidly growing genetic modification capabilities of bacteria, the use of unconscious agents and the lack of application in food safety systems.

According to the findings, 90 (30%) of the adult bee samples, 84 (28%) of the larval stages and 54 (19%) of the honey samples were positive for *S. aureus*. In contrast to Packer et al.[34] Dixon [35] reports that *S. aureus* has been destroyed in honey due to the antimicrobial effect of honey. The positivity in honey samples was significantly lower than the bee and larvae samples. One possible reason for this result is the low water activity and pH values of honey. In this study, a positive correlation was found between *E. coli* and *S. aureus* microbiological parameters. These microorganisms, which are transmitted especially as a result of personnel contamination, may be an indicator that the hygiene criteria are not complied sufficiently.

Clostridium botulinum was one of the other bacteria examined in our study. Infant botulismis is the most serious disease caused by the consumption of *C. botulinum* agent in honey. When the agents are contaminated the hives, they can form infant botulism in the dead larvae [36,37]. In this study, *C. botulinum* were detected in both bees, larvae and honey samples as in many studies around the world. While a high level of *C. botilnum* was found in a study in Lithuania, it was found in a much lower level in Serbia [38,39].

The most important fungal infection in honey bees is defined as Ascosphaeriosis (Chalkbrood Disease) [40]. It is reported that chalkbrood disease is very common in the world and in our country and seriously damages the production of bee products [41]. Although the findings obtained from our study are lower than the rates stated by Soysal and Gürcan [42], it has been observed that the agent has continued its existence in the region for at least 9 years and threatened hive and bee health. One of the possible reasons is that the agent is resistant to environmental conditions and can produce spore. Another possible reason is the suitability of environmental and hive conditions in terms of the easily reproducible agent in humid environments.

Aspergillus flavus and *Aspergillus fumigatus*, which are the most common agents of stone disease, were also studied.

No meaningful relationship was found between *Aspergillus flavus, Aspergillus fumigatus* agents and the other factors, but significant relationships were found in the presence of bees, larvae and honey samples in statistical analyses. Kırpık et al.[43] reported in their study in the region of the Caucasus plateau that identified *Aspergillus* in the bees intestinal flora in different colonies (including live bee).

In this study, some parasitological parameters were investigated and *Nosema* spp. was one of the parameters evaluated. 56 (18.7%) of the bee samples were positive while none of the honey samples were positive for *Nosema* spp. According to the statistical analysis, no significant differences were found between *Nosema* spp. and the other parameters. There are many studies carried out in different provinces related to *Nosema*, which is very common in our country. Oğuz et al.[12] determined *Nosema* spp. spores in Van province. In other studies *Nosema* spp. spore were detected in Kırşehir and Ordu Province [2,44]. Studies in the world show that nosemosis is a serious problem not only in our country but in the whole world [45]. Although *Nosema* spores cause a serious risk to the honey producer, studies show that spores are reduced as a result of good hygiene practices [24].

Varroa spp. which was also among the parasitological parameters examined in our study was positive in some bee and larva samples. Studies have shown that Varroosis is unfortunately common in our country and it is known as a dangerous external parasite that lives on the larvae, pupae and adults of the active honey bees, growing without showing any noticeable signs for a long time [46,47].

Acute Bee Paralysis Virus infection, which can cause paralysis and death in bees, is common in many countries around the world. Altough Anido et al.[48] found ABPV infection in their study, ABPV was not found in any materials in Chen et al.[49] study. In this sudy, ABPV was not found in any samples. BQCV and SBV which are so important in beekeeping affect larvae, pupae, adult bee and cause to severe disease and death. When studies on bees and larvae are examined, it is seen that BQCV and SBV exist in the world [11,49,50]. There was no positive samples in our results.

In conclusion, in this study in the Northern Marmara Region shows that many bacterial, fungal and parasitological agents that are risky for both bee and public health can be transmitted to bees, larvaes and honey. In order to prevent this contamination caused by inadequate hygiene and sanitation procedures and improper beekeeping practices, firstly beekeepers must be included in education programs. It is imperative that the producers and the relevant government agencies cooperate in a series of continuous measures. As a result of further studies investigating the existence of different disease factors that have not been studied in this study, methods to dealing with these factors and possible contamination sources can be determined.

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Research Article

Immunohistochemical Distributions of HGF and PCNA in the Kidneys of Diabetic and Non-Diabetic Mice

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Abstract

Diabetes mellitus is a systemic disease that causes functional disorders in various organs and systems. In this study, we investigated the immunohistochemical localization of hepatocyte growth factor (HGF) and proliferating cell nuclear antigen (PCNA) in the kidneys of streptozocin (STZ)-induced diabetic mice. Twenty-four Swiss albino mice were divided into three groups: control, sham and diabetic groups. STZ (100 mg/kg) was administered intraperitoneally (ip) for the development of diabetes. The avidin-biotin-peroxidase complex (ABC) technique was used to determine HGF and PCNA immunoreactivity. In diabetic kidney tissue, there was hydropic degeneration and irregularities on the epithelium of some proximal and distal tubules. Narrowing was observed in some of the Bowman's spaces. HGF and PCNA immunoreactivities were especially intense in the inner cortex and weak in the medulla. More intense HGF and PCNA immunoreactivities were found in the individual epithelial cells of the proximal and distal tubules. Immunoreactivities were stronger in the proximal tubules than in the distal tubules. In addition, HGF and PCNA immunoreactivities were strong in both interstitial regions and papillary ducts. HGF immunoreactivity was weaker in the diabetic group compared to the other groups. PCNA immunoreactivity generally decreased in the diabetic group but increased in the glomeruli of this group. The reason for the latter result was thought to be based on the increase of mesangial cells in the glomeruli.

Keywords: *Diabetes mellitus, HGF, Immunohistochemistry, Kidney, PCNA*

Diyabetik ve Nondiyabetik Farelerin Böbrek Dokusunda HGF ve PCNA'nın İmmunohistokimyasal Dağılımı

Öz

Diyabetes mellitus, çeşitli organ ve sistemlerde fonksiyonel bozukluklara neden olan sistemik bir hastalıktır. Çalışmamızda STZ ile diyabet oluşturulan farelerin böbreğinde HGF ve PCNA'nın immunohistokimyasal lokalizasyonu incelendi. Çalışmada 24 adet swiss albino fare kontrol, sham ve diyabet olmak üzere 3 gruba ayrıldı. Diyabet oluşumu için 100 mg/kg dozunda streptozosin intraperitoneal (ip) uygulandı. HGF ve PCNA immunoreaktivitesini belirlemek amacıyla Avidin-Biotin-Peroksidaz Kompleks (ABC) tekniği uygulandı. Diyabetik böbrek dokusunda bazı tubulus proksimalis ve distalis epitellerinde yer yer düzensizlik ve hidropik dejenerasyon görüldü. Bazı Bowman aralıklarında daralma gözlendi. HGF ve PCNA immunoreaktivitelerinin özellikle iç kortekste yoğun, medulla da ise zayıf olduğu gözlendi. Tubulus proksimalis ve distalis epitellerinde tek tek hücrelerde daha yoğun HGF ve PCNA immunoreaktivitelerine rastlandı. Tubulus proksimalislerdeki immunoreaktivitenin Tubulus distalislere göre daha yoğun olduğu belirlendi. Ayrıca HGF ve PCNA immunoreaktivitelerinin gerek tubüller arası intersitisyel bölgede gerek ise duktus papillariste yoğun olduğu tespit edildi. HGF immunoreaktivitesinin diyabetlilerde diğer gruplara göre daha zayıf olduğu gözlendi. PCNA immunoreaktitivitesinin genel olarak diyabetlilerde azaldığı ancak diyabetiklerin glomeruluslarında artmış olduğu görüldü. Bunun nedeninin ise mezengial hücrelerin artışından kaynaklandığı düşünüldü.

Anahtar sözcükler: *Diyabetes mellitus, HGF, İmmunohistokimya, Böbrek, PCNA*

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease that affects protein, fat and carbohydrate metabolism. It causes damage, dysfunction and deficiencies in various organs and systems in the long term [1-3]. DM also causes glomerular lesions in

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the kidneys, atherosclerosis in the vessels, pyelonephritis and thickening of the basement membranes [4].

Hepatocyte growth factor (HGF) was first identified as a factor that causes hepatocyte growth in the 1980s. It has also been reported to be a pleiotropic cytokine produced in the epithelial cells of various organs, such as pancreatic β cells, lungs and kidneys, and mesangial cells, such as fibroblasts, macrophages and smooth muscle cells [5]. HGF has many effects on renal tubular epithelial cells, including cell proliferation, motility, differentiation and cell stimulation [6]. HGF and its receptor, c-met, also have a role in renal development and maintenance of renal homeostasis [5,7-10]. HGF also plays a role in the mesenchymal-epithelial transition during kidney development [5,11]. In a previous study [12], it was shown that HGF injection increased PCNA gene expression in mice.

Proliferating cell nuclear antigen (PCNA) is a key protein that plays an important role in genomic DNA replication, recombination and repair. PCNA begins to be synthesized in the G1 phase of the cell cycle and reaches its highest level in the S phase [13,14]. PCNA acts as a helper of DNA polymerase δ [15,16]. PCNA also plays a role in the replication and repair mechanisms of nucleic acids [17]. PCNA, which is a protein that determines the rate of cell proliferation [18], coordinates the proteins and regulates their functions in cell division [19]. Few studies [20] have examined PCNA immunoreactivity in diabetic kidneys; however, HGF immunoreactivity in diabetic kidneys was not reported previously.

The aim of this study was to investigate the immunohistochemical distribution of both HGF, which plays an active role in proliferation and differentiation, and PCNA, which is a protein that determines the rate of cell proliferation and repair, in diabetic kidney.

MATERIAL and METHODS

Ethical Approval

Experimental applications in mice were performed with the approval of Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK: 2016/125).

Experimental Animals and Streptozotocin Applications

In this study, 24 Swiss albino mice 8-12 weeks of age were divided into three groups: control (n=8), sham (n=8) and diabetic (n=8). Mice were kept in standard cages at a room temperature of 22±2°C, a 12:12 h light-dark cycle with an average humidity of 50±5%. They were fed standard food and water *ad libitum*. Streptozotocin (STZ; Sigma, St Louis, MO, USA) dissolved in a 0.1 M citrate buffer (pH: 4.5) was intraperitoneally (ip) given to the diabetes group as a single dose of 100 mg/kg [21]. The sham group was treated with 0.1 M citrate buffer in the same way. No application was made to the control group. Blood glucose levels were measured with a handheld glucometer (Accu-Chek-Go, Roche, Switzerland) at 72 h after the STZ administration following an 8-h fast. Mice with a blood glucose level of 200 mg/dL or more were considered diabetic and added to the study [22]. On the 30th day of the experiment, body weight was measured, and kidney tissues were removed following a cervical dislocation under diethyl ether anesthesia [23].

Histological Examinations

Renal tissues were fixed in a 10% formalin solution for histological and immunohistochemical examinations. Tissues were then blocked in paraffin after routine histological procedures [24]. After that, 5-*μm*-thick sections were taken from the paraffin blocks. Crossman's staining (triple staining) and periodic acid-Schiff (PAS) staining were applied to the sections and photographed under light microscopy (Olympus BX51; Olympus Optical Co. Osaka, Japan).

Immunohistochemical Examination

The avidin-biotin-peroxidase complex (ABC) technique was used to determine the immunohistochemical distribution of HGF and PCNA in renal tissue. After deparaffinization and rehydration, the sections were incubated for 10 min in 3% H_2O_2 to prevent endogenous peroxidase activity. After washing with PBS (phosphate buffer solution) to reveal the antigenic sites of tissues, sections were incubated for 10 min in a 0.1 M citrate buffer (pH: 6.0) solution in a microwave oven (800 Watt). After washing again with PBS, sections were incubated in Ultra V Block serum (UltraVision Detection System Large Volume AntiPolyvalent, HRP [RTU], Thermo Scientific TP-125-HL) for 10 min to prevent nonspecific binding. After washing with PBS, the sections were incubated with anti-HGF antibody and anti-PCNA antibody (HGF: Santa Cruz: sc7949, 1:400, PCNA: Abcam: ab18197, 1:1000 dilution) for 1 h at room temperature. The sections were then washed with PBS and incubated for 30 min with biotinylated secondary antibody (UltraVision Detection System Large Volume AntiPolyvalent, HRP [RTU], Thermo Scientific TP-125-HL). After washing again with PBS, sections were incubated for 30 min with streptavidin horseradish peroxidase (UltraVision Detection System Large Volume Anti-Polyvalent, HRP [RTU], Thermo Scientific TP125HL). Sections were washed with PBS and the DAB-H_2O_2 technique was used for chromogen application. For a negative control, the same procedures were performed without adding the primary antibody. Hematoxylin was used for the nuclear counterstain. The prepared slides were examined under a research microscope (Olympus BX51; Olympus Optical Co. Osaka, Japan) and photographed. Grading of the immunohistochemistry results was performed to determine the immunoreaction according to intensity (0: no reaction; 1: mild reaction; 2: medium reaction; 3: very intense reaction). Slides were randomly selected from each subject (n=8 per group) and 20 proximal tubules, 20 distal tubules and 20 Malpighian bodies were also randomly selected from each subject of each group. Selected regions were graded in terms of the immunoreaction intensity.

The cortex and medulla of each subject were also graded in terms of the immunoreaction intensity. Scoring results were analyzed statistically to evaluate the differences between the groups.

Statistical Analysis

The data were analyzed using the SPSS 16.0 program, one-way analysis of variance (ANOVA) tests, multiple comparisons and Duncan's tests. Differences between groups were considered significant when P<0.05.

RESULTS

Blood Glucose and Body Weight

There was no significant difference between the sham and control groups in terms of blood glucose levels. However, the blood glucose level was significantly higher in the diabetic group than in the other two groups (P<0.05; *Table 1*).

There was a significant decrease in body weight in the diabetic group compared with the other groups (P<0.05). There was also a significant decrease in body weight of the diabetic group on the day 15th and day 30th of the study (P<0.05; *Table 2*).

Histological Results

The renal tissues had a histologically normal structure in the control and sham groups *(Fig. 1-A)*. In addition to the lymphocyte infiltration *(Fig. 1-B)*, irregular and hydropic degeneration were observed on the epithelial tissues of some proximal and distal tubules of diabetic mice kidneys *(Fig. 1-C)*. In the diabetic group, there was hyperemia in the glomerular capillaries and renal vessels of some Malpighian bodies, and a narrowing in some Bowman's spaces were also seen *(Fig. 1-D)*. In addition, the PAS+ reaction was stronger in the diabetic group than in the control and sham groups *(Fig. 1-E,F)*.

Immunohistochemical Results

Specific HGF immunoreactivity was observed in renal tissues of all groups. Immunoreactivity was observed in the capsule surrounding the kidney. In particular, more intense HGF immunoreactivity was detected in the inner cortex *(Fig. 2-A,B)*. Moderate immunoreactivity was observed in the Malpighian bodies. In addition, HGF immunoreactivity was seen in both the proximal and distal tubules *(Fig. 2-C)*. While HGF immunoreactivity was very weak in the distal tubules, it was stronger in the proximal tubules. Especially in individual cells in the epithelium of proximal tubules, intense cytoplasmic HGF immunoreactivity was detected *(Fig. 2-C,D)*. HGF immunoreactivity, which was observed in individual cells in the proximal tubule, was observed in fewer cells in the diabetic group *(Fig. 2-D)*. HGF immunoreactivity was also observed in the basement membranes of the proximal and distal tubules *(Fig. 2-C)*. However, intense HGF-positive cells were observed in the intertubular region. This immunoreactivity was found to be cytoplasmic in some of the cells and both cytoplasmic and nuclear in a few cells. In addition, intense HGF immuno-reactivity was observed in the blood vessel endothelium. A very weak immunoreactivity was detected in the collecting ducts and Henle's loops in the medulla, while an intense immunoreactivity was observed in the papillary ducts *(Fig. 2-A,B,E)*. In general, HGF immunoreactivity was weaker in the diabetic group than in the control group (P<0.05; *Fig. 2-A,B; Table 3)*. No immunoreactivity was detected in the negative control slide *(Fig. 2-F)*.

Table 1. *Statistical analysis of blood glucose level (mg/dL) between groups*

Groups	N	Blood Glucose Level (mg/dL)				
		0. Day	3. Day	15. Day	30. Day	P
Sham	8	109.8±10.7^{aA}	111.5±11.1^{aA}	113.5±11.7^{aA}	116.4±11.2^{aA}	0.617
Control	8	118.6±18.9^{aA}	108.2±10.7^{aA}	111.5±9.5^{aA}	117.2±11.5^{aA}	0.295
Diabetic	8	106.3±7.7^{aA}	291.1±20.7^{bB}	330.7±30^{bcB}	387.4±41.5^{cB}	0.000

^{a,b,c} At the same line, different superscript letters mean significant differences (P<0.05); ^{A,B} At the same column different superscript letters mean significant difference (P<0.05)

Table 2. *Statistical analysis of body weight (gr) between groups*

Groups	N	Body Weight (g)				
		0. Day	3. Day	15. Day	30. Day	P
Sham	8	42.49±2.94^{aA}	40.51±3.20^{aA}	41.76±2.88^{aA}	40.15±3^{aA}	0.271
Control	8	42.11±2.86^{aA}	40.27±2.82^{aA}	40.19±2.85^{aA}	39.09±2.73^{aA}	0.142
Diabetic	8	41.61±2.41^{aA}	39.17±2.13^{aA}	34.85±3^{abB}	28.76±3.9^{bB}	0.000

^{a,b} At the same line, different superscript letters mean significant differences (P<0.05); ^{A,B} At the same column different superscript letters mean significant difference (P<0.05)

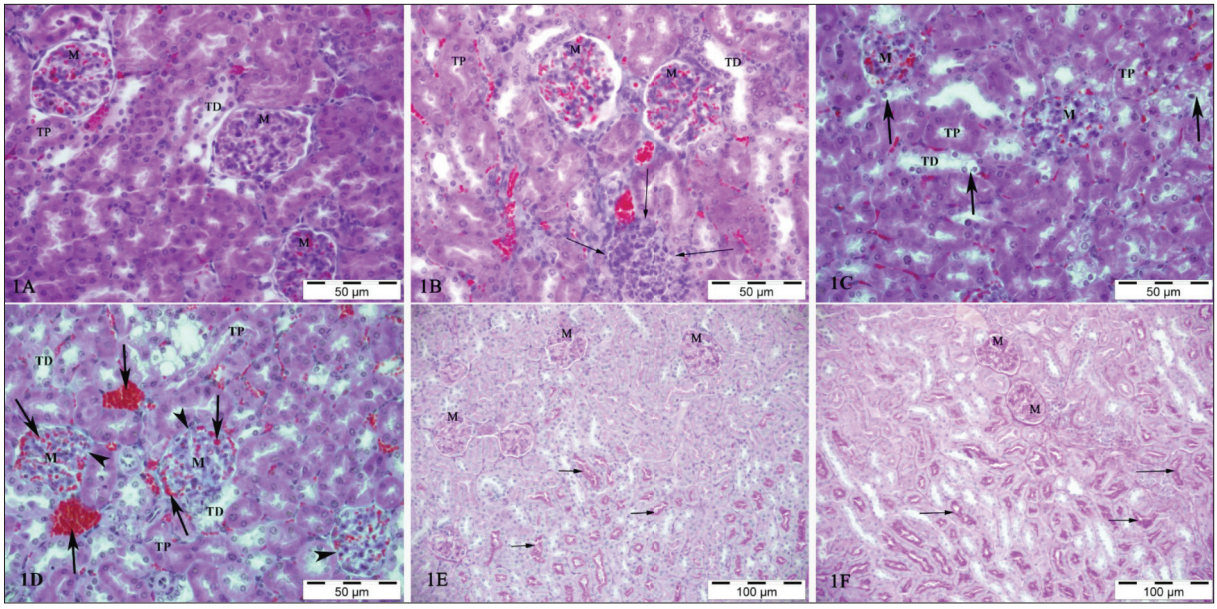

Fig 1. A) General view of kidney tissue in the control group, Triple staining; **B)** General view of kidney tissue in the diabetic group, *Arrows:* lymphocyte infiltration. Triple staining; **C)** General view of kidney tissue in the diabetic group, *Arrows:* hydropic degeneration, Triple staining; **D)** General view of kidney tissue in the diabetic group, *Arrows:* hyperemia in the glomerular capillaries and renal vessels, *Arrowhead:* Bowman's space, Triple staining; **E)** PAS staining in the control group, *Arrows:* weak PAS+ regions; **F)** PAS staining in the diabetic group, *Arrows:* strong PAS+ regions, M: Malpighian body, TP: proximal tubule, TD: distal tubule

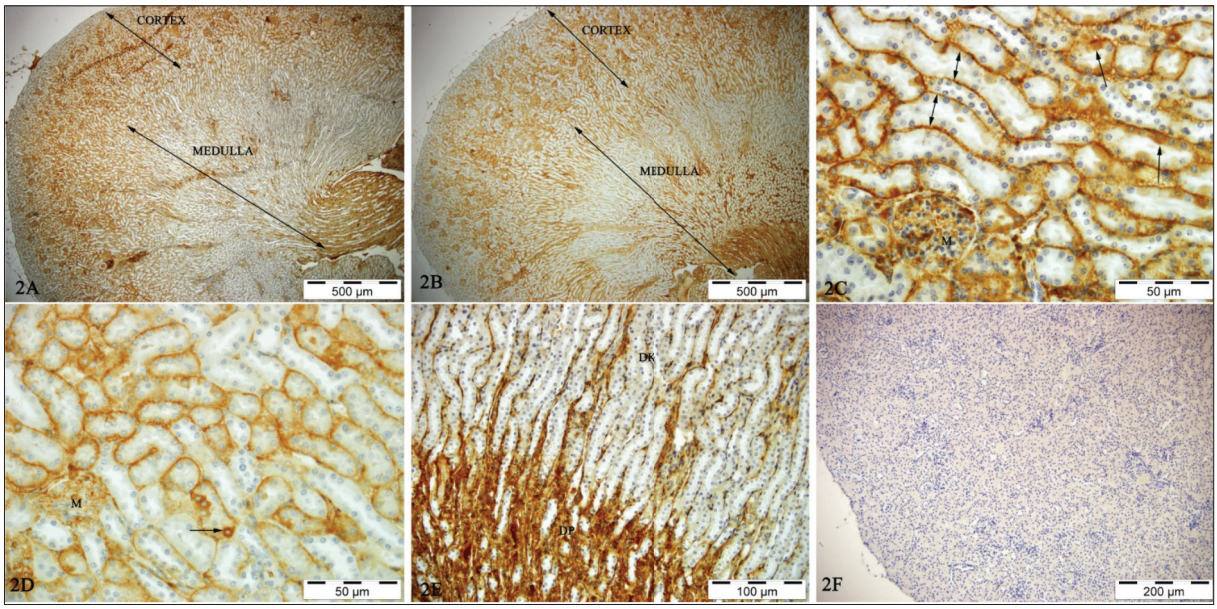

Fig 2. A) General view of HGF immunoreactivity in the control group; **B)** General view of HGF immunoreactivity in the diabetic group; **C)** Control group, M: Malpighian body, *Arrows:* strong immune positive cells, *Bidirectional arrow:* HGF immunoreactivity in the basal membrane; **D)** Diabetic group, M: Malpighian body, *Arrow:* strong immune positive cells; **E)** HGF immunoreactivity in the medulla of the control group, DK: collecting duct, DP: papillary duct, F) negative control

Proliferating cell nuclear antigen immunoreactivity was observed in the kidney capsule in all groups. PCNA immunoreactivity was strong, especially in the inner cortex, and very weak in the medulla *(Fig. 3-A)*. It was also observed that the weak immunoreactivity was found in the distal tubules and more intense immunoreactivity in the proximal tubules. The number of PCNA-positive immunoreactive cells in tubules was less in the diabetic group than in the control and sham groups *(Fig. 3-A,B)*. In addition, intense PCNA immunoreactivity was observed in both the intertubular region and papillary ducts. PCNA immunoreactivity was also observed in the blood vessel endothelium and was more intense in the Malpighian bodies in the diabetic group than in the control and sham groups *(Fig. 3-C,D)*. Although, PCNA immunoreactivity was stronger in the Malpighian bodies of the diabetic group,

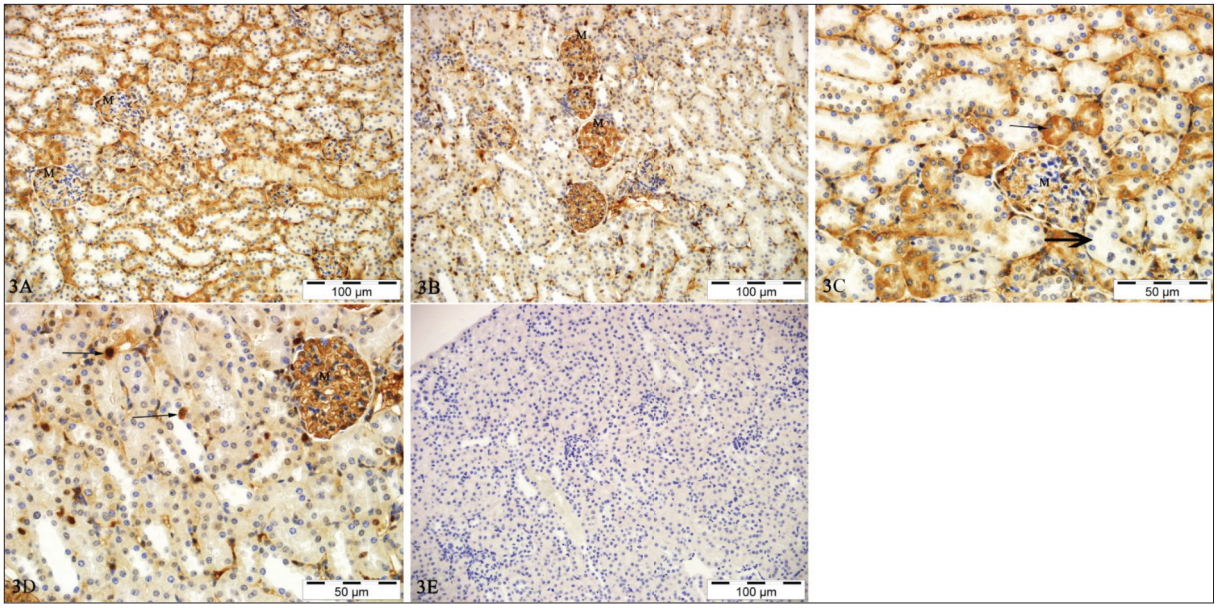

Fig 3. A) General view of PCNA immunoreactivity in the control group, M: Malpighian body; **B)** General view of PCNA immunoreactivity in the diabetic group, M: Malpighian body; **C)** Control group, M: Malpighian body, *Thin arrow:* proximal tubule, *Thick arrow:* distal tubule; **D)** Diabetic group, M: Malpighian body, *Arrows:* strong immune positive cells, **E)** Negative control

Table 3. *Statistical analysis of HGF immunoreactivity between groups*

Parts of Kidney	N	Min.	Max.	Control	Sham	Diabetic	P
				Mean±SD	Mean±SD	Mean±SD	
Cortex	8	1	3	2.70±0.46[a]	2.62±0.49[a]	1.70±0.55[b]	0.000
Medulla	8	0	2	0.58±0.50[a]	0.54±0.53[a]	0.28±0.43[b]	0.000
Malpighian body	160	1	3	2.68±0.55[a]	2.64±0.56[a]	1.65±0.65[b]	0.000
Proximal tubule	160	1	3	2.60±0.60[a]	2.51±0.60[a]	1.79±0.70[b]	0.000
Distal tubule	160	0	3	1.44±0.56[a]	1.44±0.56[a]	0.75±0.46[b]	0.640

[a,b] At the same line, different superscript letters mean significant differences (P<0.05) (0: no reaction, 1: mild reaction, 2: medium reaction, 3: very intense reaction)

Table 4. *Statistical analysis of PCNA immunoreactivity between groups*

Parts of Kidney	N	Min	Max	Control	Sham	Diabetic	P
				Mean±SD	Mean±SD	Mean±SD	
Cortex	8	0	3	2.37±0.51[a]	2.25±0.46[a]	0.62±0.51[b]	0.000
Medulla	8	0	2	0.50±0.53[a]	0.51±0.53[a]	0.49±0.54[a]	1.000
Malpighian body	160	1	3	1.25±0.43[a]	1.12±0.33[a]	2.02±0.76[b]	0.000
Proximal tubule	160	0	3	2.01±0.47[a]	2.00±0.46[a]	0.77±0.41[b]	0.000
Distal tubule	160	0	1	0.23±0.42[a]	0.18±0.39[a]	0.21±0.38[a]	0.462

[a,b] At the same line, different superscript letters mean significant differences (P<0.05); 0: no reaction, 1: mild reaction, 2: medium reaction, 3: very intense reaction)

PCNA immuno-reactivity was generally weaker in the diabetic group than in the control and sham groups in renal tissues (except for the Malpighian bodies). Generally, PCNA immunoreactivity decreased in the diabetes group (P<0.05; *Fig. 3-B,D*; *Table 4*). No immunoreactivity was seen in the negative control slide *(Fig. 3-E)*.

DISCUSSION

In this study, we aimed to investigate the effects of DM on kidney histology and immunohistochemical distribution of HGF and PCNA in diabetic and non-diabetic kidneys.

In STZ-induced diabetes studies [23,25], it has been reported that body weights in diabetic groups decrease compared to control groups. On the other hand, Al-Malki and El-Rabey [23] reported that there was no significant difference in terms of body weights in the first week of a diabetic group, but there was a significant weight loss in diabetic groups compared to the control groups at the 2nd, 3rd and 4th weeks. In the present study, body weights decreased in the diabetic group, similar to other studies [23,25].

In the mouse experimental diabetes model, it has been reported that there are degenerations of Bowman's capsule, the glomerular and tubular basement membranes and tubular dilatation [26]. Al-Malki and El Rabey [23] reported that DM caused constriction of the glomerular basement membrane in the kidney, and a hydropic degeneration within glomerular cells and tubular epithelial cells. The findings of the present study were consistent with previous findings [26,27], as the tubular structures of kidney tissues in the diabetic group exhibited irregular structures and different sizes of cellular nuclei. Al-Malki and El-Rabey [23] reported hydropic degeneration in the glomeruli and tubules of diabetic rats. In our study, hydropic degenerations were observed in some proximal and distal tubules. We thought this may be related to the STZ dose and the duration of the experiment.

Normally, HGF is produced at low levels by the liver, spleen and kidney in our bodies. Tashiro et al.[28] reported that HGF mRNA was expressed in the thymus, kidney, lungs, brain tissue, and the liver of rats. In experimental studies with HGF injections, it has been reported that HGF reduces renal fibrosis in obstructive nephropathy, increases tubular cell proliferation by inhibiting apoptosis, and reduces TGF-β1, which plays a major role in tubulointerstitial fibrosis [29,30]. Previous studies [31,32] have determined that serum HGF levels are affected by the presence and severity of certain diseases and the presence of DM complications. Wolf et al.[33] studied the immunohistochemical localization of HGF in human and rat tissues and reported that HGF immunoreactivity was strong in the distal tubules and collecting ducts in the kidney, and weak in the proximal tubules and Henle's loops. In addition, HGF immunoreactivity in the proximal tubules is mostly concentrated at the margins of the microvilli, and completely negative in the glomeruli [33]. In our study, HGF immunoreactivity was more intense, especially in the inner cortex region, while it was moderate in the collecting ducts in the medulla and intense in the papillary ducts. In addition, HGF immunoreactivity was strong in the Malpighian bodies and cytoplasmic HGF immunoreactivity was observed in very few cells in the proximal and distal tubule epithelium. To our knowledge, no studies have examined HGF immunoreactivity in diabetic kidneys. However, it has been reported that the HGF gene slows the progression of diabetic nephropathy [34], recovers renal functions in chronic renal disease [35] and high glucose levels suppress HGF production in muscle cells [36]. On the other hand, another study [37] has reported that HGF serum levels increase in patients with DM. In our study, HGF immunoreactivity was weaker in the diabetic group compared to the control and sham groups. We thought that decreased HGF immunoreactivity in diabetic kidneys may be due to tissue disorders.

Foley et al.[38] reported that PCNA immunoreactivity is found in the nuclei and cytoplasm of tubular epithelial cells in the kidney tissue. They also reported that PCNA immunoreactivity is present in mesangial cells in Malpighian bodies [38]. Gross et al.[20] have determined that PCNA immunoreactivity is more intense in the glomeruli of diabetic rats than in non-diabetic rats. However, they also reported that the number of PCNA-positive cells in the tubulointerstitial area were significantly higher in the diabetic group [20]. In our study, intense PCNA immunoreactivity was observed in the glomeruli of the diabetic group, whereas the PCNA immunoreactivity in the tubular epithelium was very weak in the diabetic group compared to the non-diabetic group. A decrease in the number of podocytes [39] and an increase in the number of mesangial cells have been reported in DM [40]. PCNA is used as a marker of mesangial cell growth [18]. In our study, we think that the increase in PCNA immunoreactivity in the glomerulus of a diabetic kidney may be based on increasing mesangial cells.

In conclusion, we observed structural disorders in renal tissue and decreased HGF immunoreactivity in diabetic mice. PCNA was generally decreased in diabetic kidneys but increased in the glomeruli of diabetic kidneys. This may be caused by a proliferation of mesangial cells in the glomeruli.

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Research Article

Phylogenetic Grouping and Antimicrobial Resistance Profiles of *Escherichia coli* Isolated from Calves in Xinjiang, China

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Abstract

The widespread multidrug-resistant *Escherichia coli* strains have caused a severe challenge to animal health and the development of breeding industries. The purpose of this study was to investigate the phylogenetic grouping and antimicrobial resistance profiles of *E. coli* isolated from diarrheic calves in Xinjiang province, China. In this study, a total of 379 *E. coli* strains were isolated from 379 rectal swab samples of diarrheic calves. They were further analyzed their phylogenetic groupings by multiplex PCR, and were clustered into four phylogenetic groups, A (36.1%), B1 (17.4%), B2 (15.6%), and D (30.9%). All *E. coli* isolates were tested for their susceptibility to 15 antimicrobial agents by Kirby-Bauer (KB) method. The isolates showed the highest resistance rates against ampicillin (64.9%), followed by streptomycin (59.4%), tetracycline (53.8%), sulfamethoxazole/trimethoprim (50.9%), chloramphenicol (45.6%), kanamycin (44.1%) and enrofloxacin (42.0%). *E. coli* isolates exhibited lower resistance to ceftazidime (15.0%) and polymyxin (12.6%). The resistance genes *bla*$_{TEM}$, *bla*$_{OXA}$, *mcr-1*, *strA-strB*, *aadA*, *tet*(A), *tet*(B), and *tet*(C) were detected in 68.3% (168/246), 27.2% (67/246), 14.6% (7/48), 51.1% (115/225), 24.9% (56/225), 51.5% (105/204), 44.6% (91/204), and 7.8% (16/204) of *E. coli* isolates, respectively. These results demonstrate that prevalent multi-drug resistance and high level of antimicrobial resistance genes exist among *E. coli* from Xinjiang diarrheic calves and pose a potential public health concern.

Keywords: *Escherichia coli, Phylogenetic grouping, Antimicrobial resistance, Resistance genes, Calf*

Çin'in Sincan Bölgesindeki Buzağılardan İzole Edilen *Escherichia coli*'nin Filogenetik Gruplandırması ve Antimikrobiyal Direnç Profili

Öz

Yaygın çoklu ilaç dirençli *Escherichia coli* suşları, hayvan sağlığı ve üretim endüstrilerinin gelişimi için ciddi bir zorluk yaratmaktadır. Bu çalışmanın amacı, Çin'in Xinjiang eyaletindeki ishalli buzağılardan izole edilen E. coli'nin filogenetik gruplandırma ve antimikrobiyal direnç profilini araştırmaktı. Çalışmada, 379 adet ishalli buzağıdan alınan rektal sıvap örneğinden toplam 379 *E. coli* suşu izole edildi. Filogenetik gruplar ayrıca çoklu PCR ile analiz edildi ve A (%36.1), B1 (%17.4), B2 (%15.6) ve D (%30.9) olarak dört gruba kümelendi. Tüm *E. coli* izolatları, Kirby-Bauer (KB) yöntemiyle 15 antimikrobiyal maddeye karşı duyarlılıkları açısından test edildi. İzolatlar ampisiline karşı en yüksek direnç oranını gösterirken (%64.9), bunu streptomisin (%59.4), tetrasiklin (%53.8), sülfametoksazol/trimetoprim (%50.9), kloramfenikol (%45.6), kanamisin (%44.1) ve enrofloksasin (%42.0) izledi. *E. coli* izolatları seftazidime (%15.0) ve polimiksine (%12.6) daha düşük direnç gösterdi. *E. coli* izolatlarında direnç genleri *bla*$_{TEM}$, *bla*$_{OXA}$, *mcr-1*, *strA-strB*, *aadA*, *tet(A)*, *tet(B)* ve *tet(C)* sırasıyla %68.3 (168/246), %27.2 (67/246), %14.6 (7/48), %51.1 (115/225), %24.9 (56/225), %51.5 (105/204), %44.6 (91/204) ve %7.8 (16/204) olarak belirlendi. Bu sonuçlar, Xinjiang bölgesindeki ishalli buzağılardan elde edilen *E. coli* suşlarında yaygın çoklu ilaç direnci ve yüksek düzeyde antimikrobiyal direnç genlerinin bulunduğunu ve potansiyel bir halk sağlığı sorunu teşkil ettiğini gösterdi.

Anahtar sözcükler: *Escherichia coli, Filogenetik gruplama, Antimikrobiyal direnç, Direnç genleri, Buzağı*

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INTRODUCTION

Antimicrobial resistance is a serious threat to both animal breeding and human health [1]. It is estimated that more than 50% of the world's antibacterials are used in husbandry industry [2], and among them, nearly 90% of antibacterial agents have been used for prophylaxis or growth promotion [3]. Due to the long-term misuse of antibiotics in economically important animals, bacterial resistance to drugs has become an increasingly severe issue [4,5] as increased incidence of antibiotic-resistant infections coupled with a declining antibiotic pipeline is creating a global public health threat [1,6].

Escherichia coli is the most common type of abundant bacteria in human and animal intestines. Some serotypes are pathogenic and can cause diarrhea, meningitis, urinary tract infections, sepsis, or pneumonia in humans and animals [7,8]. Due to the extensive use of antibiotics in veterinary clinics, it is easy for *E. coli* to evolve resistance to drugs and become a reservoir for antibiotic resistance and resistance genes [9,10]. In recent years, numerous studies have been reported on the drug resistance of *E. coli* in cattle, including resistance phenotypes and genotypes, and also the impact of antibiotics on the selection of resistance genes [11-14]. Furthermore, drug-resistant *E. coli* isolates may not only threaten veterinary clinical treatment of infections, but also possibly spread to human via the food chain, thus posing a challenge to public health [15,16].

The Xinjiang Uygur Autonomous Region, with 1.66 million km^2, is situated in northwestern China and borders Russia, Kazakhstan, and other Central Asian countries. This region is one of the major pastoral areas in China with well-developed animal husbandry industry and an estimated cattle population of 4.2 million (Xinjiang Statistical Yearbook, 2016, C832.45-54). Therefore, the aim of this study was to clarify the phylogenetic grouping, antimicrobial resistance profiles and resistance genes of *E. coli* isolates collected from calves in Xinjiang.

MATERIAL and METHODS

Ethics Statement

This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Scientific Research Department of Xinjiang Academy of Agricultural and Reclamation Sciences (protocol approval number: XJNKKXY-AEP-038). This study did not involve any endangered or protected animal species. Individual oral/written informed consent for the use of samples was obtained from all the animal owners.

Sample Sources and Antimicrobial Use Histories

From May 2016 to May 2017, a total of 379 rectal swab samples were collected from 1 to 6-month-old diarrheic calves in six large-scale dairy farms and one cattle farm located in different districts (Urumqi, Wujiaqu, Changji, Shihezi, Kuitun) in Xinjiang, China, along with geographic and cattle industry representatives.The most commonly used drugs for calves in these cattle farms were: ampicillin, streptomycin, tetracycline and sulfonamides.

Isolation and Identification of E. coli Strains

After adding 2 mL of 0.85% saline to the collection tubes, the rectal swab samples were vortexed for 10 min at room temperature and allowed to stand for 5 min, according to a previously reported protocol with minor modifications [17]. Next, 10 µL supernatant was taken and inoculated on MacConkey agar (Difco Laboratories, Detroit, MI, USA) for overnight culturing at 37ºC. One colony per sample was selected for pure culturing. The suspected *E. coli* colonies were first identified by biochemical tests (Tianhe, Hangzhou, China), and they were further confirmed based on the VITEK 2 Automatic microbial analysis system (VITEK® 2 Compact 30) and 16S rRNA PCR and sequencing *(Table 1)*. The confirmed *E. coli* isolates were selected for further investigation.

Phylogenetic Grouping of E. coli Isolates

The isolated *E. coli* was identified and grouped using the triple PCR method [18]. The groups were determined based on the presence or absence of *chuA* and *yjaA* genes, as well as an unknown DNA fragment (TspE4.C2). Primers *(Table 1)* used in this assay were synthesized by Beijing Genomics Institute (BGI). PCR products were analyzed by 1% agarose gel electrophoresis and recorded by a gel imaging system and the amplicons were sequenced.

Drug Susceptibility Test

The drug susceptibility test was conducted following the Kirby-Bauer (KB) method recommended by the Clinical and Laboratory Standards Institute [19]. The bacteria were collected with a sterile loop, suspended in peptone water, and incubated at 37ºC for 2 h. The turbidity of the suspension was adjusted to 0.5 McFarland's standard (1.5×10^8 CFU/mL). The suspension was then spread onto the surface of a cation-adjusted Mueller-Hinton agar (MHA) (AOBOX, Beijing, China) plate using sterile cotton swabs. The following 15 antimicrobial agents (Oxoid, Basingstoke, England) were included in the assay: ampicillin (AMP) (10 µg), cephalexin (LEX) (30 µg), cefotaxime (CTX) (30 µg), ceftazidime (CAZ) (30 µg), streptomycin (STR) (10 µg), gentamicin (GEN) (10 µg), kanamycin (KAN) (30 µg), amikacin (AMI) (30 µg), tetracycline (TET) (30 µg), doxycycline (DOX) (30 µg), chloramphenicol (CHL) (30 µg), polymyxin B (POL) (300 IU), norfloxacin (NOR) (10 µg), enrofloxacin (EN) (10 µg), and sulfamethoxazole/trimethoprim (SXT) (23.75/1.25 µg). Test results were interpreted based on the criteria recommended by the M100, 28th edition of the CLSI (Wayne, PA, United States) (Clinical Laboratory Standards Institute) [19]. The *E. coli* strain ATCC 25922 was used for quality control.

Table 1. *The oligonucleotide sequence and predicted sizes used in the PCR*

Primer Name	Primer Sequence (5'-3')	Target Gene	Size (bp)	Reference
16S-F	GCGGACGGGTGAGTAATGT	16S rRNA	200	This study
16S-R	TCATCCTCTCAGACCAGCTA			
ChuA-F	GACGAACCAACGGTCAGGAT	chuA	279	[17]
ChuA-R	TGCCGCCAGTACCAAAGACA			
YjaA-F	TGAAGTGTCAGGAGACGCTG	yjaA	211	[17]
YjaA-R	ATGGAGAATGCGTTCCTCAAC			
TspE4C2-F	GAGTAATGTCGGGGCATTCA	TSPE4.C2	152	[17]
TspE4C2-R	CGCGCCAACAAAGTATTACG			
bla(TEM)-F	TTGGGTGCACGACTGGGT	bla_{TEM}	503	[12]
bla(TEM)-R	TAATTGTTGCCGGGAAGC			
bla(PSE)-F	CGCTTCGGGTTAACAAGTAC	bla_{PSE}	419	[12]
bla(PSE)-R	CTGGTTCATTTCAGATAGCG			
bla(OXA)-F	AGCAGCGCCAGTGCATCA	bla_{OXA}	708	[12]
bla(OXA)-R	ATTCGACCCCAAGTTTCC			
mcr-1-F	CGGTCAGTCCGTTTGTTC	mcr-1	309	[19]
mcr-1-R	CTTGGTCGGTCTGTAGGG			
tet(A)-F	GCTACATCCTGCTTGCCTTC	*tet*(A)	210	[20]
tet(A)-R	CATAGATCGCCGTGAAGAGG			
tet(B)-F	TTGGTTAGGGGCAAGTTTTG	*tet*(B)	659	[20]
tet(B)-R	GTAATGGGCCAATAACACCG			
tet(C)-F	CTTGAGAGCCTTCAACCCAG	*tet*(C)	418	[20]
tet(C)-R	ATGGTCGTCATCTACCTGCC			
strA-strB-F	TATCTGCGATTGGACCCTCTG	strA-strB	538	[21]
strA-strB-R	CATTGCTCATCATTTGATCGGCT			
AadA-F	GCAGCGCAATGACATTCTTG	*aadA1* or *aadA2*	282	[22]
AadA-R	ATCCTCGGCGCGATTTTG			

Detection of Resistance Genes by PCR Assay

Bacterial genomic DNA was extracted according to the genome DNA extraction kit manufacturer's instructions (OMEGA Bio-tek Inc., Norcross, GA, USA), and was used as template for PCR analysis. For ampicillin-resistant *E. coli*, triple PCR was used to detect three β-lactam-resistant genes: bla_{TEM}, bla_{OXA}, and bla_{PSE} [12]. For polymyxin-resistant *E. coli, mcr-1* was detected by PCR [20]. For tetracycline-resistant *E. coli*, multiplex PCR was used to detect three tetracycline-resistant genes, *tet*(A), *tet*(B), and *tet*(C) [21]. For streptomycin -resistant *E. coli*, duplex PCR was used to test two aminoglycoside-resistant genes: *strA-strB* and *aadA* [22,23]. The target gene amplified by PCR was ligated with vector pMD19-T (TaKaRa, Dalian, China) and transformed into *E. coli* DH5α competent cells, and the recombinant plasmid was sequenced (TaKaRa, Dalian, China).

Statistical Analysis

Epi Info version 7.2 (CDC) was used to perform statistical analysis. Comparison of drug resistant differences in the four phylogenetic groups (A, B1, B2, D) of *E. coli* was conducted by the $\chi 2$ –test. $P<0.05$ was considered statistically significant.

RESULTS

E. coli Isolation and Phylogenetic Characterization

A total of 379 *E. coli* strains (100% isolation rate) were isolated from calve rectal swab samples. Isolated strains were further identified and grouped by checking their PCR products with gel electrophoresis. There were three specific bands observed, 279 bp, 211 bp and (or) 152 bp, corresponding to *chuA*, *yiaA* and the DNA fragment TspE4.C2. These strains were distributed differently among the four phylogenetic groups *(Table 2)* by comparing PCR bands with the positive strains *(Fig. 1)*. group A, B1, B2 and D accounted for 36.1% (137/379), 17.4% (66/379), 15.6% (59/379) and 30.9% (117/379).

Table 2. *Phylogenetic clustering of E. coli isolated form calves*

Phylogenetic Group(s)	No. of Isolates (%) by Origin
A	137 (36.1)
B1	66 (17.4)
B2	59 (15.6)
D	117 (30.9)

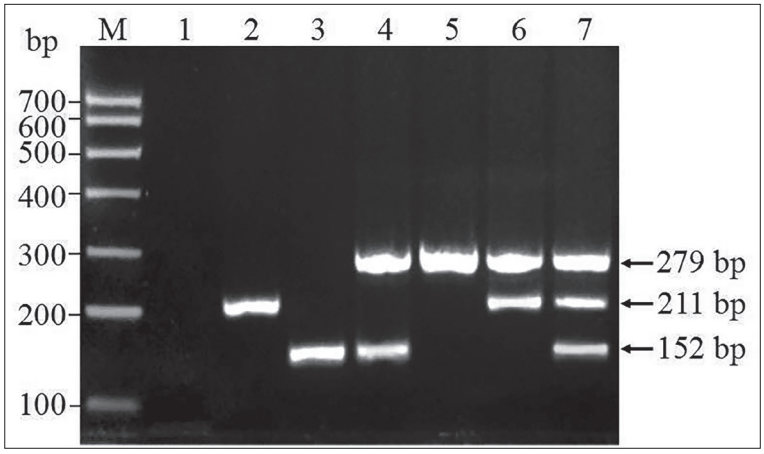

Fig 1. Phylogenetic grouping for *E. coli* isolates based on Triplex PCR method. Each combination of *chuA* and *yjaA* gene and DNA fragment TSPE4.C2 amplification allowed phylogenetic grouping of a strain. Lane M, contained markers. Lane 1 and 2, group A [*(chuA-, yjaA-,* TspE4.C2-) and *(yjaA+, chuA-,* TspE4.C2-)]; lane 3, group B1 [*chuA-, yjaA,* TspE4.C2+]; lanes 6 and 7, group B2 [*(chuA+, yjaA+,* TspE4.C2-) and (*chuA+, yjaA+,* TspE4.C2+)]; lane 4 and 5, group D [(*chuA+,yjaA-,*TspE4.C2-) and (*chuA+, yjaA-,* TspE4.C2+)]

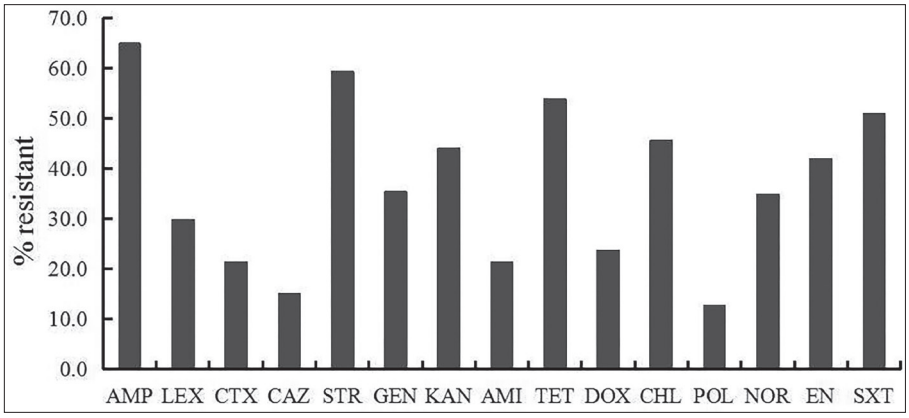

Fig 2. The antimicrobial resistance of *E. coli* isolates. AMP: Ampicillin, LEX: Cephalexin, CTX: Cefotaxime, CAZ: Ceftazidime, STR: Streptomycin, GEN: Gentamicin, KAN: Kanamycin, AMI: Amikacin, TET: Tetracycline, DOX: Doxycycline, CHL: Chloramphenicol, POL: Polymyxin B, NOR: Norfloxacin, EN: Enrofloxacin, SXT: Sulfamethoxazole/Trimethoprim

Antimicrobial Susceptibility

Of the 379 *E. coli* strains, 64.9% (246/379) were resistant to ampicillin, which was the highest rate from the 15 antibiotics tested, followed by streptomycin (59.4%), tetracycline (53.8%), sulfamethoxazole/trimethoprim (50.9%), Chloramphenicol (45.6%), Kanamycin (44.1%), Enrofloxacin (42.0%), Gentamicin (35.4%) and Norfloxacin (34.85%). Additionally, 29.8%, 23.8%, 21.4%, 21.4%, 15.0%, and 12.6% *E. coli* isolates exhibited resistance to cephalexin, doxycycline, amikacin, cefotaxime, ceftazidime, and polymyxin B, respectively *(Fig. 2, Table 3)*.

The *E. coli* from different phylogenetic groups showed different resistance to the 15 different kinds of antibiotics. Groups A and D had relatively higher resistance rates, and group B2 showed the most susceptibility to antibiotics *(Table 3)*.

Resistance Gene Profiles from Different Resistance Phenotype of E. coli Strains

Most of the 379 *E. coli* strains had different resistance genotypes *(Fig. 3, Table 4)*. Among ampicillin-resistant strains, 91.5% (225/246) carried either bla_{TEM}, or bla_{OXA} gene, or both and. no bla_{PSE} gene was detected. Among tetracycline-resistant strains, 94.1% (192/204) had one or two of the genes *tet*(A), *tet*(B) and *tet*(C). Among streptomycin-resistant strains, 70.2% (158/225) carried the *strA-strB* or *aadA* gene, or both. Among polymyxin-resistant strains, 14.6% (7/48) had the *mcr-1* gene.

DISCUSSION

In recent years, with the development of a large-scale cattle industry in China, the incidence of cattle diseases has continued to rise, it turns to be an essential issue to understand the antibiotic resistance situation among cattles in order to provide better anti-bacterial therapy and rational use of antibiotics. Antibiotics are extensively used in animal husbandry to prevent common bacterial diseases or promote livestock growth. The antimicrobial resistance has emerged as a serious threat to both the cattle industry and public health [24]. In our study, 64.9% of *E. coli* isolates were resistant to ampicillin, and more than 50% of isolates showed resistance against streptomycin, tetracycline and sulfamethoxazole/trimethoprim, Coincidentally, these four antimicrobial agents were widely used in the local cattle farm, suggesting that antimicrobial agents used in cattle have driven the emergence and abundance of resistance.

In the United States, among *E. coli* strains taken from cattle, resistance rates have been shown to be 23.7% for tetracycline, 10.5% for sulfamethoxazole/trimethoprim, and 9.5% for streptomycin [25]. In Germany, drug resistance rates of *E. coli* from calves were 65.9% for tetracycline, 59.0% for amoxicillin, 56.5% for sulfamethoxazole/trimethoprim, and

Table 3. *Antimicrobial sensitivity of different phylogenetic groups of E. coli isolates*

Classes	Antibacterial Agents	Number of Resistant Isolates (Percentage of Resistance %)				
		A (n=137)	B1 (n=66)	B2 (n=59)	D (n=117)	Total (n=379)
Beta-lactams	*Ampicillin*	84 (61.3 %)	39 (59.1%)	32 (54.2%)	91 (77.8%)	246 (64.9)
	Cephalexin	37 (27.0%)	18 (27.7%)	16 (27.1%)	42 (35.9%)	113 (29.8)
	Cefotaxime	29 (21.2%)	13 (19.7%)	12 (20.3%)	27 (23.0%)	81 (21.4)
	Ceftazidime	20 (14.6%)	8 (12.1%)	7 (11.8%)	22 (18.8%)	57 (15.0)
Aminoglycosides	*Streptomycin*	79 (57.6%)	36 (54.5%)	29 (49.2%)	81 (69.2%)	225 (59.4)
	Gentamicin	44 (32.1%)	20 (30.3%)	18 (30.5%)	52 (44.4%)	134 (35.4)
	Kanamycin	55 (40.2%)	29 (43.9%)	18 (30.5%)	65 (55.6%)	167 (44.1)
	Amikacin	30 (21.9%)	12 (18.2%)	11 (18.6%)	28 (23.9%)	81 (21.4)
Tetracyclines	*Tetracycline*	67 (48.9%)	35 (53.0%)	32 (54.2%)	70 (59.8%)	204 (53.8)
	Doxycycline	29 (21.2%)	13 (19.7%)	12 (20.3%)	36 (30.7%)	90 (23.7)
Phenicols	*Chloramphenicol*	58 (42.3%)	25 (37.9%)	21 (35.6%)	69 (58.9%)	173 (45.6)
Polypeptides	*Polymyxin B*	15 (10.1%)	8 (12.1%)	4 (6.7%)	21 (17.9%)	48(12.6)
Quinolones	*Norfloxacin*	42 (30.7%)	22 (33.3%)	16 (27.1%)	52 (44.4%)	132 (34.8)
	Enrofloxacin	51 (37.2%)	27 (40.9%)	2 0(33.9%)	61 (52.1%)	159 (42.0)
Sulfonamides	*Sulfamethoxazole/Trimethoprim*	66 (48.1%)	32 (48.5%)	21 (35.6%)	74 (63.2%)	193 (50.9)

Table 4. *Detection of resistance genes from different resistance phenotypes of clinical isolates of Escherichia coli*

Phenotype	Resistance Gene	No. of Isolates (%)
Ampicillin (n=246)	bla_{TEM}	158 (64.2)
	bla_{OXA}	57 (23.2)
	bla_{TEM} & bla_{OXA}	10 (4.1)
	No gene detected	21 (8.5)
Tetracycline (n=204)	*tet*(A)	85(41.7)
	tet(B)	74 (36.3)
	tet(C)	13(6.4)
	tet(A)+*tet*(B)	17 (8.3)
	tet(A)+*tet*(C)	3 (1.5)
	No gene detected	12 (5.9)
Streptomycin (n=225)	*strA-strB*	102 (45.3)
	aadA	43 (19.1)
	strA-strB+aadA	13 (5.8)
	No gene detected	67 (29.8)
Polymyxin B (n=48)	*mcr-1*	7 (14.6 %)
	No gene detected	41 (85.4 %)

52.4% for streptomycin [13]. In France, the drug resistance rates of *E. coli* from calves was 79.8% for tetracycline, 68.0% for sulfa drugs, 61.0% for amoxicillin, and 60.1% for streptomycin [14]. The overall rates of drug-resistant *E. coli* in this study were higher than those in the United States but lower than them of Germany and France [13,14,25].

In this study, *E. coli* isolates were divided into four phylogenetic groups, A (36.1%), B1 (17.4%), B2 (15.6%) and D (30.9%). It has been reported that B2 and D are highly pathogenic groups [18,26], and different hosts from different regions carry distinct *E. coli* groups [27,28]. Rodriguez et al.[29] found that more group A (38%) and D (28.1%) and less group B2 (18.5%) were identified among 524 avian pathogenic *E. coli* isolates from the United States. Tetsuo Asai et al.[30] demonstrated that group B2 *E. coli* from chickens only appeared in isolates from diseased chickens. Studies in Brazil and Japan showed that healthy cattle and pigs carried more groups A and B1 *E. coli* while no group B2 [26]. Extensive antibiotic use can lead to antibiotic pressure on bacterial evolution in that niche, and selection will be directed toward to success of the most resistant pathogens [31]; Simultaneously, during colonization and infection, the most virulent pathogens will be the most successful and will therefore be the most likely to survive. Our results showed that the highly pathogenic groups D were more severely resistant than symbiotic strains and low pathogenic groups *(Table 3)* suggesting that their resistance might be related to their pathogenicity.

The resistance genes are usually located on chromosomes and mobile genetic elements [32,33], and the transference of these mobile genetic elements is an important reason for increasing numbers of multi-drug-resistant bacteria [34,35]. Among 9 genes we analyzed, our samples showed positive to bla_{TEM}, bla_{OXA}, while negative to bla_{PSE}. As comparison, bla_{TEM} and bla_{PSE} instead of bla_{OXA} were detected in *E. coli* from Canadian calves [36]. The detection rate of the *mcr-1* gene was 14.6% in this study, which is higher than that of Belgian bovine *E. coli* (11.5%) [37], but lower than that of French bovine *E. coli* (20.5%) [38]. The tetracycline resistance was mainly encoded by *tet*(A) and *tet*(B) genes, wherein *tet*(A) (51.5%) had higher prevalence than *tet*(B) (46.1%); this result is similar to the studies by Guerra et al.[12] and Van

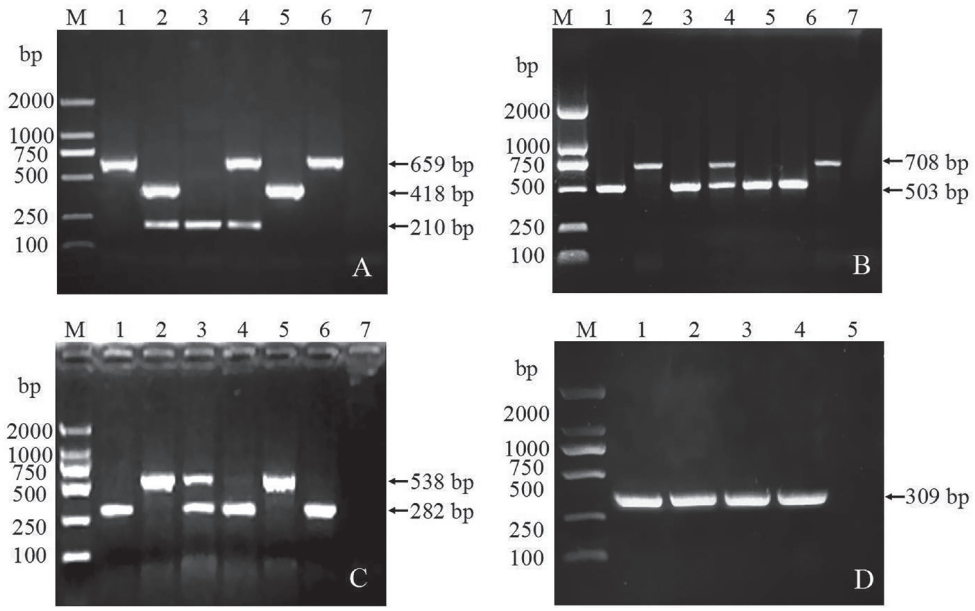

Fig 3. PCR detection of antimicrobial resistance genes in *E. coli* isolates from calves. **A:** PCR amplification of *tet*(A), *tet*(B) and *tet*(C) genes; 1-6: *tet*(A) (210 bp), *tet*(B) (659 bp) and *tet*(C) (418 bp); 7: Control negative; M: DNA Marker DL-2000; **B:** PCR amplification of bla_{TEM1}, bla_{PSE1} and bla_{OXA1} genes; 1-7: bla_{TEM1} (503 bp) and bla_{OXA1} (708 bp); 8: Control negative; M: DNA Marker DL-2000; **C:** PCR amplification of *strA-strB* and *aadA* genes; 1-6: *strA-strB* (538 bp) and *aadA* (282 bp); 7: Control negative; M: DNA Marker DL-2000; **D:** PCR amplification of *mcr-1* gene; 1-4: *mcr-1* (309 bp); 5: Control negative; M: DNA Marker DL-2000

et al.[39]. Additionally, in streptomycin-resistant *E. coli*, the *strA-strB* gene was the most common detected resistance determinant, which is consistent with previous studies [22].

In conclusion, antimicrobial resistance profiles and phylogenetic grouping of the *E. coli* clinical strains isolated from Xinjiang calves were clarified. The antibiotic resistance rates were high in diarrheal calves in Xinjiang. Therefore, the possibility of transmission of *E. coli* from calves to humans, particularly those in highly pathogenic group, can not be excluded. Also, further studies are needed to elucidate the risk of transmission to humans by analyzing the clonal relationship in *E. coli* from calves and humans.

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Conflict of Interests Statement

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

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Research Article

Comparison of Culture and PCR for Detection of Field Isolates of Bovine Milk Mollicutes

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Abstract

Mycoplasma mastitis raises significant concerns in the dairy industry worldwide. The study objective was to develop an accurate and rapid screening method for identification of field isolates of *Mycoplasma* and *Acholeplasma* species and investigate relative merits of conventional microbial culture versus PCR-based method for detecting *Mycoplasma* and *Acholeplasma* species in bovine milk. A total of 368 milk samples collected at individual cow level from a single dairy farm in South Australia, 192 (52%) tested positive for mollicutes using a conventional culture-based method. DNA extracted directly from milk and used for amplification through specifically designed universal mollicutes PCR-based method. Of them, 269 (73%) tested positive. Sequencing results of 30 positive samples targeting the 16S rRNA gene, showed five different mollicutes species involved, including *Acholeplasma laidlawii, Acholeplasma axanthum, Mycoplasma arginini, Mycoplasma bovirhinis, Mycoplasma bovis.* According to these results, species-specific PCR was conducted on all samples. DNA amplifications using species-specific PCR yielded 256 (70%) positive mollicutes samples. The developed universal PCR demonstrated best concordance with species-specific PCR (Cohen's Kappa = 0.747±0.031). Co-infection by two or more of the above-mentioned mollicutes showed highest prevalence. It is recommended surveying mollicutes using the universal PCR used in this study. The PCR system used in this study showed significant rapidity and sensitivity compared to the conventional bacteriological culture method for screening *Mycoplasma* and *Acholeplasma* species in dairy herds.

Keywords: *Mycoplasma, Mastitis, Dairy cattle, Acholeplasma*

Sığır Sütü Mollekütlerinin Saha İzolatlarının Tespiti İçin Kültür ve PCR Yöntemlerinin Karşılaştırması

Öz

Mycoplasma mastitisleri dünya çapında süt endüstrisinde önemli sorunlara yol açmaktadır. Çalışmanın amacı, *Mycoplasma* ve *Acholeplasma* türlerinin saha izolatlarının tanımlanması için doğru ve hızlı bir tarama yöntemi geliştirmek ve inek sütünde *Mycoplasma* ve *Acholeplasma* türlerinin saptanması için PCR tabanlı yönteme karşı geleneksel mikrobiyal kültürün göreceli değerlerini araştırmaktı. Güney Avustralya'daki tek bir süt çiftliğinden toplanan toplam 368 bireysel süt örneğinden 192 (%52)'sinde geleneksel kültür bazlı yöntem ile mollikût varlığı pozitif bulundu. Doğrudan sütten ekstrakte edilen DNA, özel olarak tasarlanmış evrensel mollikût PCR bazlı yöntemle amplifikasyon için kullanıldı. Bu örneklerden 269'u (%73) pozitif bulundu. 16S rRNA genini hedefleyen 30 pozitif örneğin sekanslama sonuçlarına göre, *Acholeplasma laidlawii, Acholeplasma axanthum, Mycoplasma arginini, Mycoplasma bovirhinis, Mycoplasma bovis* olmak üzere beş farklı mollikût türü belirlendi. Bu sonuçlara göre, tüm numuneler üzerinde türe özgü PCR gerçekleştirilmiştir. Türe özgü PCR kullanılarak gerçekleştirilen DNA amplifikasyonları 256 (%70) örnekte mollikût pozitif sonuç verdi. Geliştirilen evrensel PCR, türe özgü PCR ile en iyi uyumu gösterdi (Cohen's Kappa = 0.747±0.031). Yukarıda belirtilen mollikütlerin iki veya daha fazlasının birlikte enfeksiyonu yüksek prevalans gösterdi. Bu çalışmada mollikütlerin araştırılmasında evrensel PCR kullanımı önerilmektedir. Çalışmada kullanılan PCR sistemi, süt sığırcılığında *Mycoplasma* ve *Acholeplasma* türlerinin taranmasında geleneksel bakteriyolojik kültür yöntemine kıyasla önemli bir hız ve hassasiyet göstermiştir.

Anahtar sözcükler: *Mycoplasma, Mastitis, Süt sığırı, Acholeplasma*

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INTRODUCTION

Mycoplasmas and *Acholeplasma* are the smallest bacteria, belonging to the mollicutes class, andsome of these mollicutes can cause many infections in cattle. Diseases caused by *Mycoplasma* species occur worldwide causing serious problems for the dairy and beef feedlot industries and impose significant economic impact [1]. Specific concerns regarding *Mycoplasma* arise from difficulty of detection, a wide range of transmission methods, long persistence in affected herds, poor response to antimicrobials and the tendency to cause mixed infections [2,3]. *Mycoplasma* species leads to clinical, subclinical or chronic mastitis in cattle [4]. Among 200 of *Mycoplasma* species discovered, several species have been identified to be responsible for mastitis in cattle, or isolated occasionally from milk including *Mycoplasma. arginine, M. bovirhinis, M. bovigenitalium, M. bovis*, *M. californium, M. canadense, M. dispar, Mycoplasma* species bovine group 7 and F-38 [5]. *M. bovis* is the most common pathogen causing mastitis [6]. Some studies claim that *Acholeplasma* species, another genus of the mollicutes class, considered as non-pathogenic saprophyte and milk contaminant [7,8]. However, other studies have shown that involving of *A. laidlawii* in mastitis cannot be excluded [9-11]. Additionally, *Acholeplasma axanthum* has been isolated from bovine milk harvested from cattle suffering from mastitis [4]. Identification of milk *Mycoplasma* is often achieved using conventional bacteriological culture method or through serological determination methods. However, both detection methods have the significant limitation of a prolonged sampling to results timeframe [12,13]. *Mycoplasma* species can cause bovine mastitis cases either individually or as co-*Mycoplasma* infection [14]. Currently, most molecular studies focus on a single *Mycoplasma* species invader (usually *M. bovis)* and disregard potential co-infection. Few recent studies have included multiple *Mycoplasma* and *Acholeplasma* species in milk using multiplex PCR [15,16]. However, the universal PCR detailed in this work expands to few more common milk mollicutes including species not been reported previously, like. *A. axanthum* and *M. bovirhinis*. Previous studies have reported co-infections of limited variety. Current knowledge does not inform the reader of the potential combinations and their effect on milk composition and yield.

Detection of *Mycoplasma* species using 16S rRNA as a molecular marker has been previously evaluated [17-19]. The usefulness of 16S rRNA gene was demonstrated in detecting slow-growing bacteria [20]. However, most previous studies targeted species-specific oligonucleotides and disregarded co-infection of *Mycoplasma* and *Acholeplasma* species. The clinical importance of co-infection with *Mycoplasma* has been reported [9,21]. Given that mollicutes have a small genome and low G-C content [22], a sensitive, accurate and broad-species detection is required. Implementation of a rapid, reliable and affordable screening method can be used in eradication strategies of *Mycoplasma* mastitis at quarter, cattle, herd and national level.

The aim of our study was to develop a rapid, accurate and reliable screening method for identification of mollicutes, and analyse the concordance between our universal PCR, species-specific PCR and conventional bacteriological culture isolation and identification in bovine milk samples from a single commercial dairy farm in South Australia.

MATERIAL and METHODS

Samples Collection

Milk samples were collected aseptically in sterile 50 mL tubes at individual cow level from a single commercial dairy farm near Mount Gambier/South Australia. Cows had high somatic cell counts and the farm had experienced repeated failure of mastitis treatment at the time of sampling. A total of 368 milk samples were collected from each functional quarter. Milk samples were kept on ice and sent immediately to the laboratory at the School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia. Milk samples were subjected to conventional *Mycoplasma* culture, and remaining sample contents were frozen at -20°C and retained for molecular analysis.

Mollicutes Culture

Milk samples were subjected first to bacterial pre-enrichment process following the procedure described previously [23,24]. Detection of mollicutes colonies were performed using a stereomicroscope (Olympus SZ30, Vic, Australia) at 10x magnification. Positive culture samples were counted when there is growth on the mollicutes agar plate of at least one *Mycoplasma*-like colony [25]. The process of axenization of mollicutes was carried out by selecting 3-5 colonies from each plate then subcultured into the enriched *Mycoplasma* broth (Oxoid, Australia) and inoculated under the same conditions. When there was change of colour of phenol red indicator in *Mycoplasma* broth to yellow, the subculture onto a fresh broth and agar was carried out.

DNA Extraction

The DNA extraction was carried out directly, either from frozen milk or enriched samples and all tests were repeated on both type of samples. After thawing milk samples at ambient temperature, 2 mL of each milk sample was centrifuged at 8.000 g for 20 min to remove supernatant fat and excess liquid. The enriched samples in broth were used directly for DNA extraction. DNA was extracted using QIAmp DNA extraction kit (Qiagen, Germany) following the manufacturer's instructions. Genomic DNA concentration measurement was carried out using Nanodrop l000c (Thermofisher Scientific Inc., Waltham, MA, USA).

PCR Probes and Protocol

In our study, five different primers pairs were used for five separated PCR reactions. The universal primers, Mol-F: GGCGAAYGGGTGAGTAACAC and Mol-R: CATHG YCTTGGTRRGCYNTTA were designed targeting 16S rRNA gene at genus-level and generate amplicon (180 bp). Multiple sequence alignment of 16S rRNA gene was conducted on number of cattle-associated *Mycoplasma* and *Acholeplasma* using Clustal Omega software [26]. A block containing highly variable region, flanking by two conserve regions in upstream and downstream of the sequences, was selected. Based on general criteria for primer designing, forward and reverse primers were selected from conserved region of multiple blocks. The accuracy of the different primer sets for different blocks was checked by ATCC strains, PCR and sequencing. *Acholeplasma laidlawii* (Sabin) Edward and Freundt (ATCC® 23206-MINI-PACK™) and *Mycoplasma bovis* (ATCC® 25025™) were used as positive controls. *M. bovis*-specific 16S rRNA primers (442 bp), composed of PpSM5-1: 5′-CCAGCTCACCCTTATACATGAGCGC-3′ and PpSM5-2: 5′-TGACTCACCAATTAGACCGACTATTTCACC-3′ were used for *M. bovis* detection [12]; while the other three primers for *A. laidlawii*, *M. arginini* and *M. bovirhinis* were previously published elsewhere and cited in our previous work [9]. PCR reactions were carried out in 25 µL containing 0.25 µL Taq DNA polymerase (Bioline, UK), 5 µL of 5x reaction buffer (Bioline, UK), 1 µL (0.5 µM) of each forward and reverse primers (AGRF, Australia), 1 µL (approximately 20 ng) of template, and 16.75 µL of DEPC-treated water. The negative control was prepared from the same reagents of Master Mix (Bioline, UK), except DNA template, and the volume was compensated with DEPC water [27]. DNA was amplified for 35 PCR cycles conditions using T100™ Thermal Cycler (Biorad thermocycler, Australia), and consisted of pre-heating activation for 5 min at 95ºC, denaturation at 95ºC for 30 sec, annealing at 60ºC for the universal primer, *M. bovis* and A. *laidlawii*; 55ºC for *M. arginini* and 64ºC for *M. bovirhinis,* and primer extension at 72ºC for 45 sec. The final extension step was performed at 72ºC for 10 min. The PCR products were analysed by 1.5% agarose gel electrophoresis and visualised by staining with Gel Red (Biotium, US. Selected species for this study were nominated based on the 16S rRNA sequencing of the universal PCR. The same PCR methods have been done on all isolated mycoplasmas to identify the isolate in sequencing of the PCR products. All tests were carried out in duplicate. Six samples for each positive 16S rRNA PCR detected species were submitted to the Australian Genome Research Facility Ltd (AGRF, Adelaide, South Australia) for Sanger sequencing according to the method described previously [19].

Statistical Analysis

Positive results of conventional bacteriological culture method, universal PCR and species-specific PCR were reported as number and percentage. Cohen's Kappa coefficient test was used to identify the agreement between the abovementioned detection methods using (R version 3.1.1, R Development Core Team, New Zealand).

RESULTS

Of 368 milk samples collected at individual cow level from a single dairy farm in South Australia, the universal PCR used in this study showed higher prevalence of mollicutes in milk (73%) as compared to the conventional culture method (52%) *(Table 1)*. Samples were considered as positive for culture growth when at least a single colony of mollicutes was identified. PCR results were confirmed using species-specific primers (according to 16S rRNA sequencing results) for *A. laidlawii, M. arginini* and *M. bovirhinis*. Using species-specific primers, co-infection with two or more of the aforementioned mollicutes was detected in 165 (45%); *A. laidlawii* was the highest individual species detected followed by *M. bovis* and *M. bovirhinis*, *M. arginini* while *A. axanthum* had the lowest prevalence *(Fig. 1)*. In addition, 34% of samples were negative for culture and positive for either or both PCR methods (universal and species-specific). However, approximately 7% of positive samples were identified by culture but not by PCRs, 36 samples tested positive using the universal PCR, but negative using species-specific primers for *A. laidlawii*, *M. bovis*, *M. bovirhinis* and *M. arginini (Fig. 2)*. These were confirmed as *A. axanthum* via 16S rRNA sequencing. Cohen's Kappa coefficients showed good agreement between the universal PCR and species-specific PCRs and fair agreement between culture and both PCR tests *(Table 2)*.

DISCUSSION

The objective of this study was to develop an accurate, rapid, and reliable method for milk screening of *Mycoplasma* and *Acholeplasma* species and investigate the relative merits of microbiological and molecular detection of mollicutes in bovine milk.

Table 1. *Count of samples positive for detected mollicutes (Mycoplasma + Acholeplasma) species using conventional bacteriological culture method, universal PCR and species-specific PCR*

Test	Positive	Negative	Percentage
Conventional bacteriological culture method	192	176	52%
Universal PCR	269	99	73%
Species-specific PCR	256	112	70%

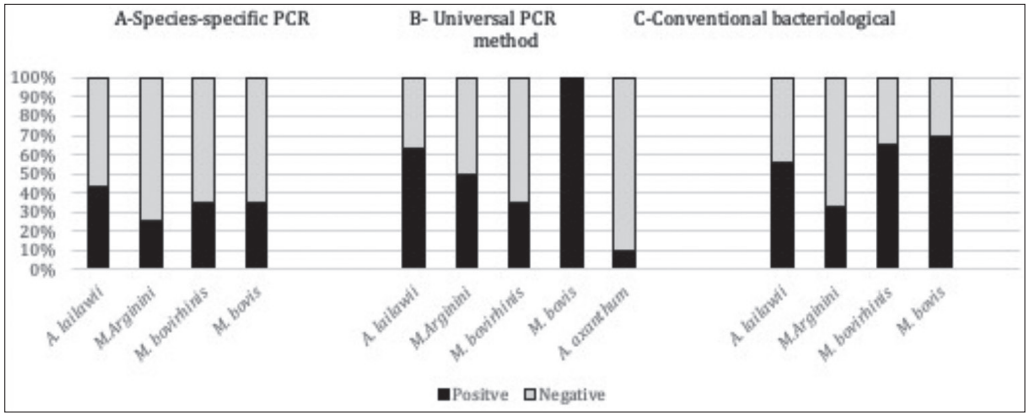

Fig 1. Percentage of positive/ negative samples detected by species-specific PCR, universal PCR and culture for each species from bovine milk samples

Table 2. *Concordance between three detecting tests for identification of Mycoplasma and Acholeplasma species from milk samples of cattle*

Tests	Concordant	Discordant	Concordant (%)	Cohen's Kappa (95% CE)	Concordance
Conventional bacteriological method versus Universal PCR	225	143	61%	0.298±0.049	Fair
Conventional bacteriological method versus Species-specific PCR	212	156	58%	0.213±0.048	Fair
Universal PCR versus Species-specific PCR	313	55	85%	0.747±0.031	Good

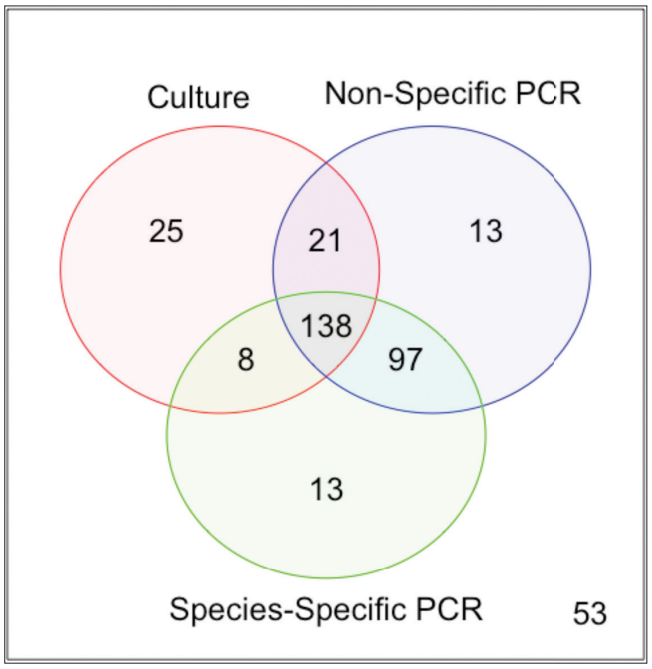

Fig 2. Venn diagram of the positive and negative samples of the three detection methods from 368 bovine milk samples from a single farm in South Australia

The studied farm had a history of treatment failure of mastitis with high somatic cell counts (~300.000 cells/ mL at bulk tank level). *Mycoplasma* mastitis has a wide range of transmission methods through milking machines and other fomites [28,29]. Another important reason for the relatively high prevalence of mastitis causing *Mycoplasma* is due to intermittent shedding of the infection from the chronic mastitic cattle [30]. It is understood that *Mycoplasma* species have the ability to form multiple micro-abscesses within the infected mammary gland leading to chronic mastitis [31]. Results of the current study can be the cornerstone for raising awareness of the consequence of mollicutes-induced mastitis for the dairy industry. The association of these mollicutes and mastitis in addition to their pathogenic significance have previously studied [9]. The study concluded that the co-infection with *Mycoplasma* and *Acholeplasma* species has similar effects on milk composition to other major mastitis pathogens [9]. Therefore, the developed universal PCR in this study is useful for milk mollicutes screening. Further research in affected herds is required to establish the current prevalence of *Mycoplasma* mastitis in Australian dairy herds. Our study found that sensitivity of mollicutes detection using the novel universal 16S rRNA amplification was significantly higher than detection using the culture-based method. Naturally, 16S rRNA demonstrates high copy numbers and low sequence diversity which can enhance sensitivity of PCR based tests [32,33]. Results of our study show that one third of samples returned negative *Mycoplasma* results for culture and positive for both PCR methods *(Fig. 1)*. This difference can be explained by the fastidious nature of *Mycoplasma* species, as failure to culture may occur due to lack of a cell wall [21], or due to involvement of multiple *Mycoplasma* species in a single case of mastitis that may have affected the growth requirements of each individual *Mycoplasma* colony. However, approximately 7% of positive samples were identified by culture, but not by PCR methods. This may be attributed to failure of DNA amplification due to existing inhibitors in milk samples or

due to failure of the developed universal 16S rRNA PCR to detect some of the species.

Although culture-based methods is still considered as a gold standard in the detection of *Mycoplasma* infection [34], the specificity of this test particularly for various genera and species of mollicutes is challenging. Morphologically, detected colonies, which grew on the specific *Mycoplasma* media, were characterised by the typical fried egg appearance. However, discrimination between different mollicutes genus and species using culture alone was not possible, *i.e.* morphology and sizes of all detected colonies appeared to be similar for most of the identified species. Indistinguishable *Mycoplasma* and *Acholeplasma* colonies have also been observed previously [7]. Hence, these authors developed biochemical and molecular differentiation techniques [7]. In this study, we have confirmed the different species using PCR/sequencing tests but not using the biochemical properties.

In conclusion, the newly developed universal PCR of 16S rRNA by this study showed significant sensitivity to detect various *Mycoplasma* and *Acholeplasma* at genus-level in milk. Direct extraction of DNA from milk for detection of *Mycoplasma* can save time and money. Consequently, implementation of our methodology may be a cornerstone for further surveys at cow, farm, regional and state level by providing a rapid, reliable and accurate method to identify milk *Mycoplasma* and *Acholeplasma* species for farmers and laboratory staff.

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Research Article

The Use of Glauconite as a Feed Additive in Broiler Nutrition and Its Effect on Growth Performance, Intestinal Histomorphology and Biomechanical Properties of Bones

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Abstract

The purpose of this study was to investigate the effects of using glauconite as a feed additive in broiler nutrition and its effect on fattening performance, intestinal histomorphology and biomechanical properties of bones in broilers. A total of 288 one-day-old male chicks were included in the study and they were randomly divided into 3 groups and these groups were divided into 8 subgroups. The trial continued for 35 days. The control group (C) was fed with basal ration while experimental groups were fed respectively with 1% glauconite (G1) and 2% glauconite (G2) added to the basal ration. The results showed that the use of different doses of glauconite in the rations did not statistically affect fattening performance. On the 21st and 35th day of the experiment, when histomorphology of ileum was examined, it was observed that villus height (VH), crypt depth (CD) and VH/CD ratio were not affected by glauconite addition. When histomorphology examination of jejeum was performed on the 35th day of the experiment, it was seen that the villus height was statistically affected. The effect of glauconite on biomechanical properties of femur and tibia bones was not statistically significant. As a result, the addition of glauconite in broiler rations did not affect the performance parameters, biomechanical properties of bones and histomorphology of the ileum, but adversely affected jejunum histomorphology.

Keywords: *Glauconite, Broiler, Intestinal histomorphology, Performance, Biomechanical properties of bones*

Broyler Beslemede Glokonitin Yem Katkı Maddesi Olarak Kullanımı ve Büyüme Performansı, Bağırsak Histomorfolojisi ve Kemiklerin Biyomekanik Özellikleri Üzerine Etkisi

Öz

Bu çalışmanın amacı, broyler beslenmede glokonitin yem katkı maddesi olarak kullanımı ve besi performansı, bağırsak histomorfolojisi ve kemiklerin biyomekanik özellikleri üzerine etkilerini araştırmaktır. Çalışmada bir günlük yaşta toplamda 288 adet civciv (Ross 308) rastgele üç gruba (Herbir grupta 96 civciv bulunmaktadır) ayrılmıştır ve bu gruplar 8 altgruba (Her alt grupta 12 civciv bulunmaktadır) ayrımıştır. Çalışma 35 gün sürmüştür. Deneme guplarına basal rasyona ek olarak %1 (G1) ve %2 (G2) glokonit ilavesi yapılırken kontrol grubu (K) bazal rasyonla beslenmiştir. Deneme sonunda rasyona farklı dozlarda glokonit ilavesinin performans parametrelerine istatistiki bir etkisi olmamıştır. Denemenin 21. ve 35. günlerinde ileum histomorfolojisi incelendiğinde, villus yüksekliği, kript derinliği ve villus yüksekliği/ kript derinliği oranının glokonit ilavesinden istatistiksel olarak etkilenmediği görülmüştür. Denemenin 35. gününde jejunum histomorfolojisi incelendiğinde, villus yüksekliği istatistiksel olarak anlamlı bulunmuştur. Denemenin sonunda tibia ve femura ait biyomekanik özellikler incelenmiştir. Glokonitin tibia ve femur ait biyomekanik özelliklere etkisi istatistiksel olarak anlamlı bulunamamıştır. Nitekim, broyler rasyonlarına glaukonit ilavesi performans, parametreleri, kemiklerin biyomekanik özellikleri ve ileum histomorfolojini etkilememiştir fakat jejunum histomorfolojisini olumsuz etkilemiştir.

Anahtar sözcükler: *Glokonit, Broiler, Bağırsak histomorfolojisi, Performans, Kemiklerin biyomekanik özellikleri*

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INTRODUCTION

Glauconite is a natural mineral with chemical formula (K, H_{20})) ($Fe^{3+,}$ Al, $Fe^{2+,}$ Mg)$_2$ [Si_3AlO_{10}] $(OH)_2$ x nH_2O) and it is a three-layer silicate mineral [1]. Glauconite is slightly acidic and capable of absorbing moisture 10 times more than ordinary sand. It consists of marine potash, silica, iron, magnesium, and lime, plus up to 30 other trace minerals. Glauconite has the property of bonding sandy soils and loosening clay soils. It is used in industrial areas such as magnetic separation and alkaline soil improvement with glauconite mineral enrichment process [2]. Element, granulometric, morphological composition of natural mineral glauconite (Belozersky field of Saratov region). Due to their sorption, physicochemical properties and environmental safety properties, they have been found to be suitable for water treatment, soil improvement and premix components for animals [3]. Minerals as ingredients in the premix are usually bentonite - montmorilloniet, glauconite, minerals of the zeolite group, limestone and others. Minerals are biostimulators of growth in poultry and livestock [4].

Glauconite is one of the clay mineral. Glauconite was historically applied as remedies in medicine. Clay-based antimicrobials consist essentially of a known antimicrobial drug or metal nanoparticles. Glauconite has the capacity to absorb various substances on the surface. Also, it can intercalate many inorganic and organic ions, replacing K^+, Ca^{2+}, etc in interlayers sites (ion-exchange capacity) [5]. Veing et al.[6] stated to be benefited from the ability to absorb many organic and inorganic ions on the surface of glauconite. Copper nanoparticles nested and adsorbed with glauconite-matrix (3-7 nm) mineral surface (30-50 nm). Superb antibacterial activity composites was reported for *Staphylococcus aureus* and *Escherichia coli*. Glauconite mineral is known to be used as dermatological and gastro-intestinal agents [6]. The clays supplemented to the ratio have the ability to bind, immobilize toxic substances in the gastrointestinal tract of animals and reduce their toxicity [7]. Clays are inherently non-toxic to environment [8]. The presence of glauconite in the gastrointestinal tract positively affects the metabolism. That is, glauconite regulates the content of intestinal fluid, electrolyte and acid-base balance and mineral metabolism. The use of glauconite in combination with probiotics in pig rations increased immune and natural organism resistance [9].

Prohibition of the use of antibiotics and other growth factor chemicals for residual release and resistance to bacteria has led to the search for alternative feed additives [10,11]. The use of glauconite clay mineral as a non-toxic and historically therapeutic use has aroused interest in its usability as a feed additive. There is no study in the literature that the glauconite mineral is used as a feed additive in any animal experiment. Due to these properties of glauconite mineral, this study was carried out to determine the effect of glauconite usage on the performance, intestinal morphology and biomechanical properties of bones in broiler feeding.

MATERIAL and METHODS

Animals, Experimental Design and Feed

This study was carried out with the permission of the Ankara University Animal Experiments Local Ethics Committee (Decision No: AU-HAYDEK /2014-23-157) report.

The glauconite mineral used in the experiment was supplied by Russia Saratov University. The particle size of the glauconite mineral used in the experiment is 60 microns. Chickens used in this study (Ross 308) was provided from a commercial hatchery (Beypiliç, Bol, Turkey). A total of 288 one-day-old male chicks (Ross 308) were included in the study and they were randomly divided into 3 groups (96 chicks in each group) and these groups were divided into 8 subgroups (12 chicks in each group). The animals were fed with corn, soy bean meal basal ration and trial continued for 35 days. The house temperature was monitored thermostatically throughout the study. The temperature, which was 32-35°C on the first day, was gradually lowered and maintained at 22°C in the last two weeks. The starter, grower, and finisher rations were given to the animals for 0-14, 15-28, and 29-35 day intervals, respectively *(Table 1)*. All rations were formulated to NRC [12] nutrient recommendations. Each subgroups were equipped with manual feeders and automatic nipple drinkers. Water and feed were given *ad libitum*. Ration treatments were as follows: C, basal diet (Control; without addition); G1, glauconite 1% and G2, glauconite 2%. Ration nutrient analyzes were performed according to AOAC [13]. The chemical composition of glauconite mineral is given in *Table 2*.

Growth Performance

In the study, live weights (LW) were recorded for each subgroup weekly. Live weight gain (LWG) was determined with the difference between these measurements. For each subgroups feed intake (FI) of animals was recorded weekly and used for the calculation of the feed conversion ratio. Performance parameters were calculated by considering the subgroup averages.

Histomorphologic Measurements

On 21th and 35th days of the study one animal from each subgroup was randomly selected for histomorphological analysis. Animals were cut off with a suitable method for the intestinal histomorphological and the biomechanical properties of bones.

The tissue samples for histomorphological examination were taken from the jejunum and ileum. In order to provide uniformity for each animal, specimens were obtained from 8 cm proximal of the Meckel's diverticula for jejunum and from 8 cm proximal of the ileocecal junction for ileum.

Table 1. *The composition of the rations used in the study (%)*[1]

Ingredient	Broiler Starter 0-14. days	Broiler Grower 15-28. days	Broiler Finisher 29-35. days
Corn	51.00	52.25	56.45
Soybean (Full fat), 38%	19.62	18.00	14.00
Vegetable oil	1.00	2.00	3.00
Soybean meal, 48%	24.00	24.00	23.00
DCP	2.40	2.00	2.00
Limestone	0.8	0.85	0.85
Bicarbonate	0.10	0.10	0.10
Salt	0.25	0.25	0.25
DL-Metiyonin	0.37	0.25	0.15
L-lizin	0.2	0.10	0
Vitamin premix[2]	0.10	0.10	0.10
Mineral premix[3]	0.10	0.10	0.10
Anticoccidial	0.06	-	-
Total	100.00	100.00	100.00
Chemical composition, calculated			
Crude protein, %	22.01	21.56	20.03
ME, kcal/kg	3099	3158	3219
Ca, %	1.01	0.92	0.91
P, %	00.5	0.44	0.44
Methionine + Cysteine, %	1.09	0.96	0.82
Lysine, %	1.44	1.33	1.14
Analysis values			
ME, kcal/kg	3131	3153	3200
Crude protein, %	23.45	21.70	19.60
Ca, %	1.04	1.00	0.93
Total P, %	0.53	0.50	0.48

[1] As-fed basis; [2] Provided per kilogram of complete diet: Vit. A, 12.000 IU; Vit. D_3, 2.500 IU; Vit. E, 40 IU; Vit. K_3, 5 mg; thiamin, 2.5 mg; riboflavin, 6 mg; pyridoxine, 5 mg; pantothenic acid, 15 mg; niacin, 25 mg; folic acid, 1 mg; biotin, 50 µg; Vit.B_{12}, 20 µg; [3] Provided per kilogram of complete diet: Cu, 5 mg; I, 1 mg, Co, 200 µg; Se, 150 µg; Fe, 60 mg; Zn, 60 mg; Mn, 80 mg. Folic Acid 1.000 mg/kg, Biotin 50 mg/kg, Copper 5.000 IU/kg, Iodine 1.000 IU/kg, Cobalt 200 mg/kg, Selenium 150 mg/kg, Iron 60.000 mg/kg, Zinc 60.000 mg/kg, Mangan 80.000 mg/kg

Table 2. *The chemical composition of glauconite used in this study (%)*

Glauconite	
Moisture	10.30
SiO_2	61.82
Al_2O_3	21.08
Fe_2O_3	3.25
CaO	2.44
MgO	2.67
CaO + MgO	5.11
K_2O	0.95
Na_2O	2.44

Each intestinal specimen (jejunum and ileum) has a size of 1 cm. Tissue samples were fixed in 10% neutral buffered formaline for 24 h and then dehydrated in graded ethanol solutions, washed with tap water, purged with xylol and embedded in paraffin, respectively. Intestinal segments were cut off the thickness of 5 µm with a microtome. Cross sections were prepared and stained with Mallory's triple stain modified by Crossman in order to determine the intestinal morphometry [14].

Villus height (VH) was determined as the area from the top of the villus to the crypt mouth. Crypt depth (CD) was measured as the area between adjacent crypt terminals [15]. Histological sections were examined using a light microscope and photographed with Leica DFC450 digital microscope camera. The images were then evaluated with ImageJ software.

Femur and Tibia Biomechanical Properties

Left femur and tibia samples were thawed at 4°C and cleaned of all tissue. Length and width of femur and tibia samples were measured using digital calliper. Afterwards bone samples were stored at -20°C for further analyses. Femurs and tibias were subjected to the three-point bending tests until problem occurred, with Instron 5944 testing frame (Instron, Norwood, MA, USA). The loading rate was 5 mm/min. Spon length was 70 mm for bones. The load was applied to the midpoint of the shaft. Load versus displacement data was collected for each sample. Stiffness values were determined from the slope of the linear region of the load displacement curves. Ultimate load (UL) and displacement at ultimate load (DUL) were calculated from the load displacement curves as well. The load at which the permanent deformation of the system begins is the yield load (YL). The displacement at which the permanent deformation begins is the displacement at the yield load (DYL) [16].

Statistical Analysis

The one-way variance analysis method (ANOVA) was used for the statistical calculations of the groups and the importance of the differences between the mean values in the groups and a suitable post hoc test (Duncan) was used for the importance control of the differences between the groups. The statistical analysis was done with the SPSS software package [17].

RESULTS

The results obtained in this study are presented with appropriate tables.

Performances

On the 7th and 28th days of the experiment, the live weight value between the groups was statistically significant ($P<0.05$). Live weight gain (LWG) values between the groups were

Table 3. *Effects of dietary supplementation of glauconite on live weight, live weight gain, feed intake and feed conversion ratio in the broiler (g)*

Performance Parameters	Days	Control		G1		G2		Significance
		X	Sx	X	Sx	X	Sx	P
Live Weight, g Days	0	41.68^{a}	0.08	41.29^{ab}	0.06	41.25^{b}	0.07	0.001***
	7	149.29^{a}	2.8	145.67^{ab}	2.11	140.39^{b}	1.56	0.032*
	14	386.84	4.46	395.34	4.45	383.31	4.84	0.187
	21	818.83	7.7	799.98	10.11	809.35	7.7	0.319
	28	1445.23^{a}	13.46	1383.99^{b}	19.82	1394.69^{b}	16.67	0.040*
	35	2175.42	29.62	2089.75	36.43	2070.50	26.32	0.060
Live Weight Gain, g, Days	0-7	107.61^{a}	2.73	104.37^{ab}	2.11	99.14^{b}	1.56	0.039*
	7-14	237.54	4.37	249.67	4.09	242.91	4.74	0.175
	14-21	431.99	8.18	404.64	9.72	426.03	6.93	0.074
	21-28	626.39	10.83	584.00	17.86	585.34	13.52	0.083
	28-35	730.19	27.13	705.75	21.83	675.80	25.27	0.320
	0-35	2133.73	29.61	2048.45	36.38	2029.24	26.30	0.061
Feed Intake, g Days	0-7	140.87	2.85	119.03	15.12	135.29	3.97	0.240
	7-14	393.63^{ab}	6.96	379.93^{b}	5.96	416.72^{a}	14.44	0.047*
	14-21	587.87	27.51	628.84	15.33	656.48	7.72	0.053
	21-28	929.43	20.84	1024.66	37.03	1083.89	69.31	0.087
	28-35	1279.47	57.01	1248.80	60.96	1248.32	50.77	0.905
	0-35	3331.29	76.81	3401.29	70.97	3540.68	118.18	0.276
Feed Conversion Ratio Days	0-7	1.31	0.02	1.12	0.14	1.36	0.04	0.156
	7-14	1.66^{a}	0.03	1.52^{b}	0.02	1.71^{a}	0.03	0.001***
	14-21	1.35^{b}	0.05	1.55^{a}	0.02	1.54^{a}	0.03	0.005***
	21-28	1.48^{b}	0.03	1.77^{a}	0.10	1.84^{a}	0.09	0.014*
	28-35	1.76	0.09	1.78	0.10	1.86	0.09	0.752
	0-35	1.56^{b}	0.03	1.66^{ab}	0.06	1.74^{b}	0.05	0.055

*Statistically not significant (P>0.05). The mean (X) and standard error (Sx) values of 8 subgroups in each group. $^{a, b, c}$ Differences between the mean values of different letters in the same row are statistically significant. * P<0.05, *** P<0.01; Groups; C: Control, G1: 1% glauconite, G2: 2% glauconite*

statistically significant in the first week of the experiment (P<0.05). Feed intake (FI) values between the groups were statistically significant in the second week of the experiment (P<0.05). In the mentioned week, the lowest feed intake value belongs to the G1 group. Feed intake was affected positively by the use of low-dose glauconite in the second week of the trial. The feed conversion ratio (FCR) between the groups at the 2nd, 3rd and 4th weeks of the experiment was found to be statistically significant (P<0.01). In the mentioned weeks, the lowest feed conversion ratio value belongs to the control group. At the end of the experiment, the use of different doses of glauconite in rations did not statistically affect the LW, LWG, FI and FCR. The effect of glauconit use at 1% and 2% as feed additive in broiler rations during the experiment on the parameters of LW, LWG, FI and FCR is given in *Table 3*.

Morphological Measurement of the Jejunum and the Ileum

On the 21st and 35th day of the experiment, when histology of ileum was examined, it was observed that VH, CD and VH/CD ratio were not affected by glauconite addition. On the 21st day of the trial, when the jejenum histomorphology was examined, it was seen that VH and VH/CD ratio values were not affected by the addition of glauconite. However, CD value was found to be statistically significant. The crypt depth of the control group was higher than those of the other groups (P<0.05). When histological examination of jejeum was performed on the 35th day of the experiment, CD and VH/CD ratio values between groups were not statistically significant. However, VH value was found to be adversely significant (P<0.05). Histomorphological measurements of jejunum and ileum at 21st and 35th day of the experiment are given in *Table 4*.

Femur and Tibia Biomechanical Properties

On the 35th day of the experiment, biomechanical properties of the femur and tibia bones from each subgroup were investigated. The effect of glauconite addition on biomechanical properties of femur and tibia bones was not statistically significant. The femur and tibia biomechanical properties on the 35th day of the trial are given in *Table 5*.

Table 4. *Effect of glauconite supplementation on histomorphology of ileum and jejunum on the 21st and 35th days of the trial (µm)*

Histomorphology Parameters	C		G1		G2		P
	X	Sx	X	Sx	X	Sx	
Ileum 21							
Ileum villus height (µm)	777.87	98.78	690.85	38.99	754.75	66.67	0.708
Ileum crypt depth (µm)	166.62	13.62	142.00	8.38	162.29	10.59	0.297
Ileum villus height/crypt depth	4.89	0.80	4.94	0.33	4.75	0.46	0.974
Jejenum 21							
Jejenum villus height (µm)	1017.33	77.20	1011.33	94.40	994.66	22.30	0.973
Jejenum crypt depth (µm)	196.27[a]	12.71	147.38[b]	6.98	167.55[ab]	11.15	0.017*
Jejenum villus height/crypt depth	5.27	0.45	6.84	0.47	6.09	0.48	0.094
Ileum 35							
Ileum villus height (µm)	938.83	64.94	92.33	69.22	913.33	21.28	0.949
Ileum crypt depth (µm)	157.66	15.77	126.77	7.14	155.83	11.81	0.168
Ileum villus height/crypt depth	6.11	0.40	7.42	0.72	6.04	0.48	0.172
Jejenum 35							
Jejenum villus height (µm)	1379.00[a]	38.20	1031.00[b]	71.22	1106.33[b]	77.12	0.004*
Jejenum crypt depth (µm)	201.33	7.89	195.50	9.32	186.00	7.03	0.426
Jejenum villus height/crypt depth	6.90	0.31	5.35	0.49	5.98	0.43	0.062

*Statistically not significant (P>0.05). The mean (X) and standard error (Sx) values of 8 subgroups in each group. Groups; C: Control, G1: 1% glauconite, G2: 2% glauconite. [a,b] Differences between the mean values of different letters in the same row are statistically significant * (P<0.05), *** (P<0.001)*

Tablo 5. *Effects of dietary glauconit treatments on femur and tibia parameters on d 35th*

Bone	Item	Dietary Treatment						
		C		G1		G2		P
		X	Sx	X	Sx	X	Sx	
Femur	Length, mm	67.33	0.39	67.00	0.32	65.98	0.69	0.141
	Width, mm	9.62	0.10	9.61	0.17	9.68	0.18	0.940
	UL, N	237.55	13.83	242.54	8.00	250.65	12.60	0.732
	DUL, mm	4.06	0.23	4.39	0.29	4.52	0.39	0.582
	YL, N	165.66	16.54	162.55	16.91	174.22	19.46	0.891
	DYL, mm	2.03	0.12	2.07	0.23	2.40	0.32	0.497
	Stiffness, N/mm	80.54	4.85	79.48	3.61	76.49	6.54	0.848
Tibia	Length, mm	92.12	0.67	91.90	0.76	91.47	0.85	0.828
	Width, mm	9.05	0.22	9.41	0.24	9.24	0.23	0.563
	UL, N	210.15	14.50	225.75	11.31	235.74	16.50	0.455
	DUL, mm	3.39	0.14	3.62	0.11	3.52	0.19	0.577
	YL, N	194.00	12.57	202.66	11.48	213.81	15.92	0.589
	DYL, mm	2.75	0.07	2.77	0.05	2.70	0.12	0.831
	Stiffness, N/mm	70.67	4.77	72.93	3.62	78.90	3.47	0.342

Statistically insignificant (P<0.05). The values show the mean (X) and standard error (Sx) of the 8 subgroups in each group. Groups; C: Control, G1: 1% glauconite, G2: 2% glauconite
Data represent mean values of 8 replicates per treatment; K: corn-soybean meal basal diet containing 0% glauconit; G1: basal diet containing 1% glauconit, G2: basal diet containing 2% glauconit; UL: Ultimate Load, DUL: Displacement at Ultimate Load, YL: Yield Load, DYL: Displacement at Yield Load

DISCUSSION

The use of clays in animal nutrition affects digestibility of nutrients, live weight gain and feed conversion rate positively [18]. In the literature research, there are few studies in which glauconite mineral is used as animal feed additive and it has not been investigated in terms of certain parameters examined in this study. In our experiment, the use of different doses of glauconite did not statistically affect the LW and LWG on the 35th day. There are many current studies using clay group minerals that support the results of our study [19,20]. However, there are many recent studies suggesting that clay group minerals have a positive effect on the performance of broiler feeding [21,22]. There are no studies on the use of glauconite in poultry nutrition. Therefore, glauconite studies in different animals were used in the discussion section. The use of glauconite in bull-calves rations increased live weight [23]. In our experiment, the use of different doses of glauconite did not statistically affect feed intake on the 35th day. It was observed that the feed intake increased between 7-14 days and the highest value was in G2. In a different study using bentonite, feed intake was reported to be increased [24]. Research results are consistent with recent articles. The use of glauconite at the level of 4% in broiler diets did not affect FI and FCR values [25]. The researchers associated that increased feed intake increase the pellet quality of bentonite by acting as a pellet binder. Similarly, a study conducted with sepiolite showed an increase in pellet quality [26]. The effect of glauconite in this direction and the related feed intake changes were not observed since this study was carried out with powder feed. Although the positive effects of glauconite application on the feed conversion ratio were observed during the experiment period, no significant effect was observed at the end of the experiment. It was reported that kaolin and zeolite will have a positive effect on feed conversation ration whereas in the same study, it was reported that bentonite increases the ratio [27]. It was stated that the feed conversation ratio will be affected positively in studies performed with different silica minerals [22]. However, different studies indicate that silica minerals do not have a significant effect on feed conversation ratio [20,24]. The results obtained can be affected by different conditions such as the quality of the clay mineral used and the feed structure. It is suggested that the mineral structure and metal oxide content of clay mineral can differentiate the results of the study [26].

Morphological changes in the small intestine, villus height (VH), crypt depth (CD) and VH/CD ratio may improve poultry performance by improving nutrient digestion and absorption [28]. When used in animal feeding, the clays caused morphological changes in the intestinal mucosa, such as an increase in villus height and an increase in crypt depth. These morphological changes increase the surface area of the gastrointestinal tract and thereby increase nutrient digestion [7]. In our study, it was observed that the addition of ration glauconite did not affect the ileum histomorphology on the 21st and 35th days of the study. In our study, the addition of ration glauconite on the 21st day of the study affected the jejunum CD. The highest crypt depth was in the control group and the lowest in the G1 group. On the 35th day of the study, the addition of ration glauconite affected the VH of the jejunum. The highest VH in the control group and the lowest G1 group. In glauconite use, high doses were found to be more beneficial for intestinal health than low dose.It has been reported that clinopilolies in different structure increased VH but had no effect on CD [29]. In a different study, the use of zeolite has been reported to increase in the length of intestinal villus [30]. In the study performed with sepiolite, an increase in the length of the duodenum villus was reported [22]. In the studies carried out with Cu-montmorillonite, an increase in villus length was formed [31]. The results obtained can be affected by different conditions such as the quality of the clay mineral used and the feed structure.

Leg abnormalities are very important problems in rapidly growing broilers, leading to economic losses and reduced welfare [32]. A study of the effect of glauconite mineral on the biomechanical properties of bones in poultry has not been found in literature review. In our study, biomechanical properties of femur and tibia bones were not affected by the addition of glauconite to the ration and no adverse effect was observed. There are studies on clay minerals and other minerals in different animals. Safari et al.[25] reported that the effect of 4% glauconite on broiler diets on the weight, length and density of the tibia was statistically insignificant. It was reported that the addition of organic trace minerals and 25-hydroxycholecalciferol to turkey rations has a positive effect on the biomechanical properties of bones [33]. The effect of different levels of boron on the laying hen rations is not insignificant on the all of tibial biomechanical properties [34]. In a different study, use of calcium soaps of animal fats in broiler feeding did not affect on the biomechanical properties of femur [35]. Although this study is compatible with our study, many studies are needed in this area. The results obtained may be affected by different conditions such as particle size, structure and dosage of the glauconite mineral used. The conflicting results might depend on the type and dose of the used clay mineral, chemical composition, coop conditions and environmental factors.

In conclusion, the use of glauconite at 1% and 2% levels in broiler rations did not affect performance parameters, biomechanical properties of bones and histomorphology of ileum but adversely affected jejunum histomorphology. In the use of glauconite, high doses were found to be more beneficial for intestinal health than low doses. There is not enough study on the use of glauconite as a feed additive in broiler feed, and studies to be carried out in different doses with glauconites in different structures will shed light on the potential of using this substance as feed additive. In

our study, it is seen that many studies are needed in order to explain the effect levels of the investigated parameters. Our study is a good literature for further studies.

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Research Article

The Levels of Trace Elements and Macrominerals in Calves with Sepsis [1]

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Abstract

The aim of this study was to determine the levels of macrominerals and trace elements in the blood of newborn calves with sepsis. The study was carried out on a total of 30 calves, aged 2-35 days old, of which 25 with sepsis and 5 healthy. In clinical examination, prolonged capillary refill time and tachypnea were observed in calves with sepsis. The levels of Cu, K, P and S in calves with sepsis were higher compared to the control group, and the levels of Na, total and ionized Ca, Fe and Zn were also lower compared to the control group. In conclusion, in this study, significant increase in K and Cu concentrations and significant decrease in Na and Ca concentrations were found in calves with sepsis. According to the results, K, Cu, Na and Ca may have important roles in the pathophysiology of sepsis.

Keywords: *Calves, Macromineral, Sepsis, Trace elements*

Sepsisli Buzağılarda İz Elementler ve Makrominerallerin Seviyeleri

Öz

Bu çalışmanın amacı, sepsisli yenidoğan buzağılarda makromineraller ve iz elementlerin seviyelerini belirlemektir. Çalışma, 2-35 günlük, 25'i sepsisli ve 5'i sağlıklı olan toplam 30 buzağı üzerinde gerçekleştirildi. Klinik muayenede sepsisli buzağılarda kapiller geri dolum süresinde uzama ve takipne gözlendi. Kontrol grubu ile karşılaştırıldığında sepsisli buzağıların Cu, K, P ve S düzeyleri yüksek, Na, total ve iyonize Ca, Fe ve Zn düzeyleri düşüktü. Sonuç olarak, bu çalışmada sepsisli buzağılarda K ve Cu konsantrasyonlarında önemli artış, Na ve Ca konsantrasyonlarında ise anlamlı azalma olduğu tespit edilmiştir. Bu sonuçlar doğrultusunda K, Cu, Na ve Ca'un sepsisin patofizyolojisinde önemli bir rolü olabileceği değerlendirildi.

Anahtar sözcükler: *Buzağı, Makromineral, Sepsis, İz elementler*

INTRODUCTION

Septicemia is the presence of a pathogenic bacterium in circulation and different organs and systems. Septicemia in newborn farm animals is usually associated with *E. coli* and *Salmonella* spp., which are significant morbitiy and mortality causes [1]. *E. coli* is a bacterium that is the most commonly isolated from blood circulation in septicemic calves [2-4], however, gram positive bacteria have been determined in 10% of septicemic calves, and polymicrobial infection in 28% [5]. Failure of passive transfer of colostral immunoglobulins is a high risk for bacterial infection [1,6,7].

For proper growth of animals and continuation of reproductive functions, inorganic elements are essential. Calcium, phosphorus, sodium, chlorine, potassium, magnesium and sulfur are found in large amounts in an organism. These elements have significant functions in body. The elements that are less needed by the organism are called trace minerals. These elements are: cobalt, copper, iodine, ferrous, manganese, molybdenum, selenium, zinc, fluorine, chromium, etc. The trace minerals are the components of some important hormones or enzyme cofactors with metalloenzyme [8,9]. The inflammatory conditions cause significant changes in the levels of trace elements and macro minerals.

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These changes are the result of the reaction of the organism to an inflammatory response [10-12]. Ranjan et al.[11] found a significant increase in blood copper level and a significant decrease in zinc level in calves with diarrhea compared to healthy calves. In addition, endotoxemia in cattle has been reported to cause a decrease in zinc and calcium levels [10]. Furthermore, hyponatremia and hyperkalemia are common findings in dehydrated and endotoxemic neonatal ruminants [13].

Significant changes in micro and macro elements were observed in patients with systemic inflammatory response syndrome (SIRS), sepsis and many critical diseases in human medicine. We assumed that sepsis in calves may cause changes in trace elements and macrominerals. Therefore, the aim of this study was to determine serum macromineral levels and trace elements in newborn calves with sepsis.

MATERIAL and METHODS

This study was conducted between 2010 and 2015 at the Large Animal Clinic, Faculty of Veterinary Medicine, Selcuk University. Five healthy and 25 calves with sepsis were used in the study. The calves were 2 to 35 days old and have different genders. Routine clinical examination of all the calves was performed. For diagnosis of sepsis, sick calves were examined in terms of parameters such as leucocytes count, body temperature, respiratory rate, suck reflex, dehydration degree, pulse, mental state, mucous membrane, capillary refill time and ability to standing. Laboratory and clinical findings as described by Aydogdu et al.[14] and Yıldız et al.[15] were used for the diagnosis of sepsis in the calves. For this purpose, calves with suspicious or present infection with SIRS criteria 2 and above were evaluated as sepsis.

Criteria for diagnosis of SIRS in calves were as follow; Leukopenia or leukocytosis (reference value, 4-12 10^3/µL), hypothermia and hyperthermia (reference value; 38.5-39.5ºC), bradycardia or tachycardia (<90 or >120 beats per minute), and tachypnea (>36 breaths per minute).

Blood sample for leucocytes count, macrominerals and trace elements analyses was collected from the *vena jugularis*. The tubes without anticoagulant were incubated at room temperature, and then their serum was removed by centrifugation for 5 min at 2500 g. Serum samples were stored at -20ºC until analyzed. Leucocyte levels in blood with K3EDTA of the calves were determined using a hematologic analyzer (Hemocell Counter MS4e, Melet Schloesing Laboratories, France). Sodium (Na), potassium (K) and ionized calcium (iCa) levels in heparinized blood were determined by a blood gas analyzer (GEM Premier Plus, Instrumentation Laboratory, Lexington, Mass). From the serum samples of the calves, calcium (Ca), boron (B), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), phosphorus (P), sulfur (S) and zinc (Zn) concentrations were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, Vista model, Varian, Australia).

This study was approved by the ethics committee of Faculty of Veterinary Medicine, Selcuk University (Approval No: 2010/052).

Statistical Analysis

Kolmogorov-Smirnov test was performed to determine the normal distribution of data. Comparisons between the groups were made by conducting independent t test for variables with a normal distribution, whilst for variables that do not show normal distribution were analyzed with the Mann-Whitney U test. Data were presented as mean values and standard errors of mean (Mean±SEM) for variables with a normal distribution and median (minimum/maximum) values for variables that do not show normal distribution. The level of statistical significance was at $P<0.05$. The SPSS software program (Version 18.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Hyperthermia or hypothermia, tachypnea, dehydration, tachycardia or bradycardia, depression, absence of sucking reflex, cooling in the extremities and in some cases, lateral recumbence were determined in calves with sepsis. In addition, capillary refill time (>2 sec) had been prolonged. Enteritis (n=18), pneumonia (n=4), intestinal obstruction (n=1), enteritis + arthritis (n=1), and pneumoenteritis (n=1) were diagnosed in calves with sepsis.

Differences in the clinical findings of sick and healthy calves are presented in *Table 1*. There was a significant increase ($P<0.05$) in respiratory rate and capillary refill

Table 1. *Clinical findings of calves with healthy and sepsis*

Parameters	Healthy Mean±SEM (n=5)	Sepsis Mean±SEM (n=25)	P Levels
Temperature (℃)	38.78±0.18	38.69±0.45	0.855
Pulse (min)	99.00±3.97	108.86±7.13	0.236
Respiratory rate (min)	34.40±3.92	60.43±6.94	**<0.001**
CRT (sec)	2.00±0.00	4.47±0.22	**<0.001**

CRT: Capillary refill time

time of the calves with sepsis compared with the control group. In addition, leukocytosis was observed in the calves with sepsis.

Macromineral and trace elements levels in sick and healthy calves are presented in *Table 2*. While blood Cu and K levels of the calves with sepsis were significantly higher (P<0.05), total Ca, iCa and Na levels were significantly lower (P<0.05) compared to the control group. Furthermore, compared to the control group, while blood Fe and Zn levels of the calves with sepsis were lower, the P and S levels were higher, no statistical difference was determined.

DISCUSSION

Due to its high morbidity and mortality, neonatal sepsis is one of the most significant health problems in cattle breeding [5,14,15]. The findings such as mild depression and losing suck reflex in the early period of sepsis are nonspecific. In sepsis, rectal temperature is variable (hypothermia or hyperthermia); however, a continuous tachycardia and even a tachypnea may develop. Furthermore, clinical symptoms related to hypotension and decreased cardiac output (prolonged capillary refill time, diminished peripheral pulses, cold extremities, decreased urine output) are evident and usually hypovolemia develops [1,4,15]. The findings for the diagnosis of sepsis according to observed hyperthermia/hypothermia, tachypnea, dehydration, tachycardia/bradycardia, depression, loss of sucking reflex, cold feeling in mouth, cooling down in extremities and in some cases, lateral recumbence, coma and prolonged capillary refill time used in this study are similar to clinical findings of sepsis *(Table 1)* stated in previous studies [1,4,14-19].

In human medicine, in sepsis and inflammatory diseases, the levels of trace elements, which have an important role in the continuation of cellular functions, stabilization of cell membranes, ensuring the efficacy of many antioxidant enzymes and the development of the immune response, and their efficacy are monitored and evaluated for prognosis and treatment [20-22]. In studies, it has been reported that in inflammation and sepsis, significant changes occur in the levels of blood copper and zinc [22-24]. Srinivas et al.[25], reported a decrease in blood Zn levels and an increase in Cu levels within a few days following infection in bacterially infected patients, and these changes persistand for weeks. An increase in serum Cu level and decrease in Zn level were determined in guinea pigs with intra-abdominal sepsis [22] and with experimental endotoxemic hamsters [26]. In studies of cattle with infectious bovine rhinotracheitis [27] and calves with diarrhea [11], a significant decrease in blood Zn level and a significant increase in copper level were determined. Endotoxemia causes negative mineral balance (hypoferremia and hypozincemia). Changes in mineral levels (such as iron and zinc) during the acute phase response help cleanse and protect the body from bacterial

Table 2. *Macromineral and trace element levels of calves with healthy and sepsis*

Parameters	Healthy n=5	Sepsis n=25	P Levels
Na (mmol/L) Mean±SEM	143.40±1.44	135.17±2.15	**0.004**
K (mmol/L) Mean±SEM	4.36±0.25	5.50±0.33	**0.013**
iCa (mmol/L) Mean±SEM	1.09±0.03	0.97±0.04	**0.031**
Ca (mg/L) Mean±SEM	136.21±2.50	119.85±3.14	**0.001**
B (mg/L) Median (min/max)	0.14 (0.10/0.23)	0.14 (0.07/0.59)	0.741
Cr (mg/L) Mean±SEM	0.06±0.01	0.06±0.01	0.805
Cu (mg/L) Mean±SEM	0.54±0.04	0.88±0.07	**<0.001**
Fe (mg/L) Mean±SEM	0.94±0.24	0.90±0.11	0.896
Mg (mg/L) Median (min/max)	69.57 (68.64/73.30)	68.23 (31.06/132.91)	0.487
P (mg/L) Median (min/max)	183.73 (177.28/194.26)	188.40 (139.36/545.15)	0.872
S (mg/L) Mean±SEM	520.35±27.89	558.95±24.95	0.323
Zn (mg/L) Mean±SEM	1.01±0.09	0.82±0.05	0.120

Na: sodium, K: potassium, iCa: ionized calcium, Ca: calcium, B: boron, Cr: chromium, Cu: copper, Fe: iron, Mg: magnesium, P: phosphorus, S: sulfur, Zn: zinc

invasion. On the contrary, an increase in blood copper level is usually seen in endotoxemia. This increase is accompanied by an increase in ceruloplasmin. Ceruloplasmin is an acute phase protein that increases from the initial stage of inflammation [1]. In this study, a significant increase in serum Cu concentration in the calves with sepsis *(Table 2)* but a decrease in Zn level compared to the control group were determined. It was considered that the increase in serum Cu level may be related to the increased level of ceruloplasmin (acute phase response), and the decrease in Zn level may originate from the increase of Zn transition from plasma to hepatocytes as a result of septicemia/endotoxemia.

Hyponatremic, hyperkalemic metabolic acidosis is usually observed in dehydrated or endotoxemic neonatal ruminants [13]. Hyperkalemia is observed as a response to metabolic acidosis [28]. Functions of Na^+-K^+-ATPase pump at physiological pH limits are optimal. Inadequacy of Na^+-K^+-ATPase pump functions during acidemia begins and this causes an increase in intracellular Na and extracellular K ions [29]. In addition, impairment of renal K excretion associated with hypovolemia due to dehydration can also lead to hyperkalemia [30,31]. Hyponatremia is one of the common findings in the calves with diarrhea [13]. In this study, compared to the control group, a significant increase ($P<0.05$) in K level, and in contrast, a significant decrease ($P<0.05$) in Na level was determined in the calves with sepsis *(Table 2)*.

Serum calcium level can be affected by endotoxicosis. Total calcium concentration drops below 2 mmol/L in the cattle with experimental endotoxicosis [10]. Furthermore, hypocalcemia is one of the most frequently observed electrolyte abnormalities in intensive care units in human medicine. In 90% of critical patients, a low total calcium concentration is reported, and the prevalence of hypocalcemia, which is measured as ionized calcium is estimated to be 15-20% [32,33]. In addition, there was a relation between hypocalcemia and an increase in mortality of patients in intensive care units [33]. In this study, a significantly decrease ($P<0.05$) in both total and ionized calcium compared to the control group may be attributed to inadequate food intake or else to other factors which affect calcium absorption (SIRS, renal failure, insufficiency of Vit D etc.).

In conclusion, in this study, significant increase in K and Cu concentrations and significant decrease in Na and Ca concentrations were found in calves with sepsis. According to the results, K, Cu, Na and Ca may have an important role in the pathophysiology of sepsis. In addition, controlled studies to monitor changes in trace element and macromineral levels during the treatment of calves with sepsis would be beneficial.

Conflict of Interests

The authors reported that there was no conflict of interest.

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Research Article

Screening of Chinese Medicinal Herbs for Anthelmintic Efficacy Against *Gyrodactylus kobayashii* (Monogenea) in Goldfish *(Carassius auratus)*

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Abstract

Monogenean infection can cause high mortality and significant financial losses in commercially farmed fish. Existing chemical drugs for the treatment of such infections often have serious drawbacks. In order to find alternative agents of chemical drugs, fourteen medicinal plants were tested for their in vivo anthelmintic activity against *Gyrodactylus kobayashii* in goldfish *(Carassius auratus)*. Ethanol extracts of *Evodia rutaecarpa, Cnidium monnieri* and *Sophora flavescens* had 100% anthelmintic efficacy at low concentrations (100, 100 and 300 mg/L, respectively), after 48 h of exposure. The ethyl acetate extract of *C. monnieri* was the most effective, with an EC_{50} value of 11.0 mg/L, after 48 h of exposure, showing 100% anthelmintic efficacy against *G. kobayashii* at 50.0 mg/L. Higher anthelmintic activity was also observed for remaining extracts of *C. monnieri* and *E. rutaecarpa* except for the water extracts of the two plants. The ethyl acetate extract of *C. monnieri* had the highest therapeutic index (TI, LC_{50}/EC_{50}) value of 31.8, and the lowest EC_{50}, which indicates that this extract was the safest to goldfish among all extracts. For the remaining extracts of *C. monnieri* and *E. rutaecarpa,* the 48-h LC_{50} values were about 10-fold higher than the corresponding EC_{50} values. This result indicates these extracts have low toxicity in goldfish. The ethyl acetate extract of *C. monnieri* was the most effective and the safest among the tested extracts. Therefore, the two plants are expected to be alternative agents to control monogenean infection.

Keywords: *Disease control, Gyrodactylus kobayashii, Chinese Medicinal Herb, Goldfish, Anthelmintic efficacy*

Japon Balıklarında *(Carassius auratus) Gyrodactylus kobayashii*'ye (Monogenea) Karşı Çin Şifalı Otlarının Anthelmintik Etkinliklerinin İncelenmesi

Öz

Monogenean enfeksiyonları, ticari olarak yetiştirilen balıklarda yüksek mortalite ve önemli finansal kayıplara neden olabilir. Bu tür enfeksiyonların tedavisi için mevcut kimyasal ilaçların genellikle ciddi dezavantajları vardır. Kimyasal ilaçların alternatif ajanlarını belirleyebilmek için on dört şifalı bitki, akvaryum balığındaki *(Carassius auratus)* Gyrodactylus kobayashii'ye karşı in vivo antelmintik aktiviteleri açısından test edildi. *Evodia rutaecarpa, Cnidium monnieri* ve *Sophora flavescens*'in etanol özleri, 48 saat maruziyetten sonra düşük konsantrasyonlarda (sırasıyla 100, 100 ve 300 mg/L) %100 antelmintik etkinliğe sahipti. *C. monnieri*'nin etil asetat özütü, 48 saat maruziyetten sonra EC_{50} değeri 11.0 mg/L ile en etkili olanıydı ve 50.0 mg/L'de *G. kobayashii*'ye karşı %100 antelmintik etkinlik gösterdi. *C. monnieri* ve *E. rutaecarpa*'nın su özütleri dışındaki özütleri için de daha yüksek antelmintik aktivite gözlemlendi. *C. monnieri*'nin etil asetat ekstresi, en yüksek terapötik indeks (TI, LC_{50}/EC_{50}) değeri 31.8 ve en düşük EC_{50}'ye sahipti, ve bu bulgu bu ekstraktın tüm ekstraktlar arasında akvaryum balığı için en güvenli olduğunu gösterdi. *C. monnieri* ve *E. rutaecarpa*'nın geri kalan ekstraktları için 48 saatlik LC_{50} değerleri, ilgili EC_{50} değerlerinden yaklaşık 10 kat daha yüksekti. Bu sonuç, bu ekstraktların Japon balıklarında düşük toksisiteye sahip olduğunu gösterdi. *C. monnieri*'nin etil asetat özütü, test edilen özütler arasında en etkili ve en güvenli olanıydı. Bu nedenle, iki bitki Monogenean enfeksiyonlarının kontrolünde alternatif ajanlar olabilir.

Anahtar sözcükler: *Hastalık kontrolü, Gyrodactylus kobayashii, Çin şifalı bitki, Japon balığı, Antelmintik etkinlik*

INTRODUCTION

Ornamental fish trade is one of the most rapidly developing areas of the aquaculture industry, and the value of international trade exports of ornamental fish has grown by an average of about 14% per year since 1985 [1,2]. The

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rapid development of the ornamental fish industry has been overwhelmed by the occurrence of large-scale parasitic diseases. Goldfish *(Carassius auratus)* is one of most popular ornamental fish worldwide, due to its easy maintenance and its attractive coloration. The most common ecto-parasites infecting goldfish are gyrodactylids [3]. Viviparous gyrodactylids have a direct life-cycle. They can directly spread among hosts by contact, and undergo continuous transmission throughout their life-cycle [4,5]. Serious infection can cause evident clinical symptoms [3]. Monogeneans can damage the fish epidermis, which results in secondary infections by other pathogenic microorganisms [6,7].

It is difficult to control monogeneans. Many chemical drugs have been used against these parasites, but bring some drawbacks (e.g., low efficacy, toxicity to host and environmental and human health problems) [8,9]. Additionally, the long-term use of chemical drugs can lead to drug resistance in parasites [10]. Recently, increasing attention has been paid to the use of traditional plant-based medicines to control diseases in aquaculture [11,12]. Zhou et al.[13] showed that herbal medicines were able to control *Gyrodactylus kobayashii (G. kobayashii)* infection in goldfish. Huang et al.[14] screened plant extracts with anthelmintic activity against *Dactylogyrus intermedius* (Monogenea) in goldfish.

In order to find safe and efficient alternatives to treat monogenean infection in aquaculture, we evaluated the anthelmintic efficacy of ethanol extracts of 14 plants against *G. kobayashii* in goldfish, using *in vivo* anthelmintic efficacy assays.

MATERIAL and METHODS

Establishment of a Goldfish-G. kobayashii Model and Animal Ethics

Goldfish weighing 3.7±0.7 g were selected from a fish farm in Anyang city, Henan province, China. All experiments complied with institutional animal care guidelines and were approved by the Animal Care Committee of Anyang Institute of Technology and Academician Workstation of Animal Disease Control and Nutrition Immunity in Henan Province (License no. SCXK(AWADCNI)2018-0001). These goldfish were fed and treated to remove all ectoparasites, as described previously [13]. A goldfish experimental infection model using *G. kobayashii* was performed according to a method described previously [15]. Uninfected fish were anesthetized with 0.02% MS-222. In order to allow parasite transfer between hosts, the caudal fins of uninfected fish were placed in contact with the caudal fins of heavily-infected fish reared in our laboratory. Experimentally-infected fish were then placed in a 1 L container. The success of the infection was determined by daily examination of the fins using a stereomicroscope. Ten days after infection, two parasites were collected from the infected fish for morphological and molecular identification according to Li et al.[16] in order to verify that the infective agent was *G. kobayashii*. Uninfected goldfish were introduced periodically into an 80 L aquarium containing infected goldfish, in order to increase the number of infected fish for the experiments.

Preparation of Plant Materials

The fourteen Chinese medicinal herbs listed in *Table 1* were purchased from a drug store and prepared according to a method described previously [13]. The herbal medicines were washed thoroughly, air-dried and oven-dried at 45°C for 48 h. In order to ensure the complete removal of water, dry plant materials were powdered, strained using a filter net of 30-40 mesh (450-600 µm), and freeze-dried at -54°C. The powder of all the medicinal plants was extracted with ethanol. The powder of *E. rutaecarpa*, *C. monnieri* and *S. flavescens* was extracted for 48 h with water, methanol, petroleum ether, or ethyl acetate; the extraction process was repeated three times. To obtain solidified crude extracts, the extract solutions were filtered, and then concentrated at reduced pressure in a vacuum rotary evaporator, until all solvents had evaporated. Solid extracts then were dissolved in dimethyl sulfoxide (DMSO) at 0.5 mg/L as stock solutions for anthelmintic efficacy assays.

Screening Experiment

The dry powder (50.0 g) of herbal medicines was extracted three times with 500.0 mL ethanol for 48 h. Ethanol filtrates were evaporated at reduced pressure in a vacuum rotary evaporator in order to obtain solidified crude extracts [14]. The solidified crude extract of each plant was dissolved in DMSO as a stock solution at 0.5 g/mL; these stock solutions were then screened for anthelmintic efficacy.

In addition, three kinds of herbal medicines *(E. rutaecarpa*, *C. monnieri* and *S. flavescens)* with higher antiparasitic activities *(Table 1)* were fractionated with different solvents (water, methanol, petroleum ether, and ethyl acetate), and then tested in anthelmintic efficacy assays. Each solid extract was dissolved in DMSO to obtain the stock solution at the concentration of 0.5 g/mL, which was used in anthelmintic efficacy assays.

In vivo Anthelmintic Efficacy Assay

The anthelmintic assay against *G. kobayashii* in goldfish was performed according to a previous method [13]. Two goldfish infected with *G. kobayashii* were placed in 20×12×10 cm tanks containing 0.5 L of the test solutions at 23±1°C for 48 h. Two control groups without extract, or with the highest percentage of DMSO, were set at the same experimental conditions. All treatments and control groups were performed using five replicates. Before manipulation, goldfish were anesthetized with 0.02% MS-222, and the parasites in the caudal fin were counted under a stereomicroscope.

Table 1. *The tested medicinal plants and the anthelmintic efficacy (AE) against Gyrodactylus kobayashii in goldfish of the ethanol extracts*

Species	Plant Part Used	Mean Intensity (±SD)	The Best AE (%)	The Concentrations of the Best AE (mg/L)	The Concentrations of Fish Died (mg/L)
Evodia rutaecarpa (Juss.) Benth.	Fruit	102.6±45.2	100	100	500
Cnidium monnieri (L.) Cuss.	Fruit	87.6±35.4	100	100	200
Sophora flavescens Ait.	Root	95.9±50.4	100	300	500
Areca catechu L.	Seed	101.8±39.6	100	150	150
Citrus reticulate Blanco	Bark	96.9±51.5	100	400	800
Mentha haplocalyx Briq.	Leaf	117.8±40.1	100	400	1000
Punica granatum L.	Bark	105.8±42.4	100	600	800
Agrimonia pilosa Ledeb.	Aerialparts	109.1±37.1	100	600	600
Omphalia lapidescens Schroet.	Sclerotium	112.3±43.4	100	800	800
Quisqualis indica L.	Fruit	82.7±30.4	94.7	800	1000
Dryopteris crassirhizoma Nakai	Root, stem and leaf	101.7±25.7	89.0	200	200
Pharbitis nil (L.) Choisy	Seed	95.9±28.5	54.4	200	200
Stemona sessilifolia (Miq.) Miq.	Root	91.1±24.0	43.3	700	700
Cynanchum paniculatum (Bge) Kitaga	Rhizome	98.1±39.1	9.9	300	300

The anthelmintic efficacy of each extract was calculated according to the following formula: $E = (L–L_t)/L\times100\%$ for $L>L_t$, $E = 0$ for $L\leq L_t$. E is anthelmintic efficacy, L is the number of *G. kobayashii* on the caudal fin before treatment, and L_t is the number of surviving parasites after the treatment. Mean anthelmintic efficacy was calculated from five replicates per treatment.

Acute Toxicity Tests

The extract with the strongest anthelmintic efficacy in *in vivo* anthelmintic efficacy assay, was tested for safety to goldfish. Acute toxicity tests were conducted in 26.5×16.5×12.5 cm tanks, containing ten healthy goldfish, and 2 L of aerated tap water at 23±1°C. Fish in tanks without plant extracts constituted the control group. Three replicates were set for the group of control fish and for the groups of fish exposed to plant's extracts. Fish mortality in each tank was recorded after 48 h. Fish were not fed during the experiment, and dead fish were continuously removed in order to avoid deterioration of water quality.

Statistical Analyses

The homogeneity of replication samples was evaluated through the Mann-Whitney U test. At the 95% confidence level, median lethal concentration (LC_{50}, LC_{90}) and median effective concentration (EC_{50}, EC_{90}) were determined using the probit analysis. The therapeutic index (TI) was calculated as LC_{50}/EC_{50}. Each statistical analysis was performed using SPSS 19.0. A value of $P<0.05$ was considered significant and $P<0.01$ was considered highly significant.

RESULTS

Anthelmintic Efficacy of Extracts Against G. kobayashii In vivo

As shown in *Table 1*, of the 14 medicinal plants selected, only the ethanol extracts of *E. rutaecarpa*, *C. monnieri* and *S. flavescens* had 100% anthelmintic efficacy at 100.0, 100.0 and 300.0 mg/L, respectively, after 48 h of exposure. Additionally, the extracts of *Citrus reticulate*, *Mentha haplocalyx*, *Punica granatum*, *Agrimonia pilosa* and *Omphalia lapidescens* showed good anthelmintic efficacy, but only at high concentrations *(Table 1)*. There was either very weak anthelmintic activity, or highly toxicity to goldfish, in the remaining ethanol extracts of the herbal medicines tested. In the control groups, the number of *G. kobayashii* increased on the caudal fin (from 98.2±25, 7 to 160.5±42.7 in the group with no extract, and from 113.1±21.5 to 150.5±31.3 in the DMSO group).

For the different extracts of *E. rutaecarpa*, *C. monnieri* and *S. flavescens*, their anthelmintic efficacy is showed in *Fig. 1, 2, 3,* and the corresponding EC_{50} and EC_{90} values are showed in *Table 2*. The ethyl acetate extract of *C. monnieri* exhibited 100% anthelmintic efficacy against *G. kobayashii* at 50.0 mg/L, and was the most effective against *G. kobayashii*, with an EC_{50} value of 11.1 mg/L, and an EC_{90} value of 28.3 mg/L, after 48 h of exposure. In addition, the anthelmintic activity against *G. kobayashii* was also determined for the methanol and petroleum ether extracts of *C. monnieri*, with EC_{50} values of 23.6 and 44.6 mg/L, and EC_{90} values of 51.0 and 84.0 mg/L, respectively. In all the extracts of *C. monnieri*, the water extract had the weakest anthelmintic efficacy of 42.7% at 1 000.0 mg/L.

The ethyl acetate extract of *E. rutaecarpa* had good anthelmintic efficacy, with EC_{50} and EC_{90} values of 24.0 and 50.3 mg/L, respectively, after a 48-h exposure. The petroleum ether and methanol extracts of *E. rutaecarpa* showed 100% anthelmintic efficacy at 150.0 and 120.0 mg/L, with EC_{50} and EC_{90} values of 71.9 and 108.3 mg/L (petroleum ether), and 40.9 and 91.2 mg/L (methanol), respectively. The water extract of *E. rutaecarpa* had the weakest anthelmintic efficacy of 25.6% at 800.0 mg/L. All

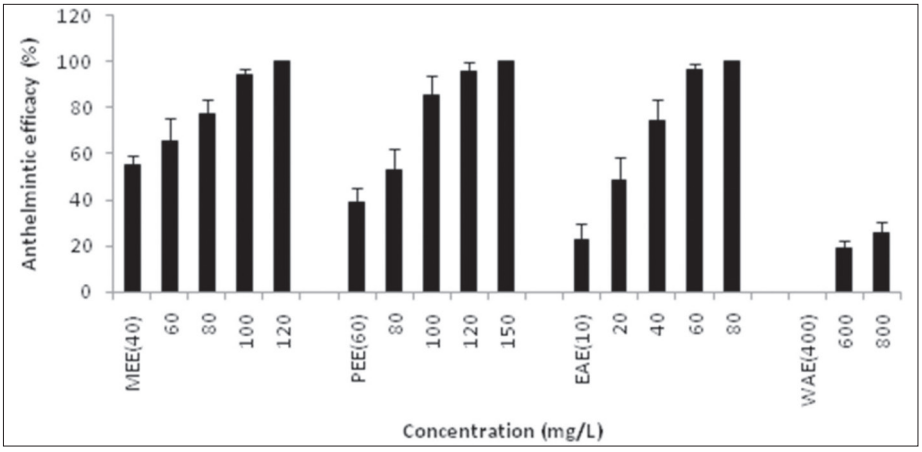
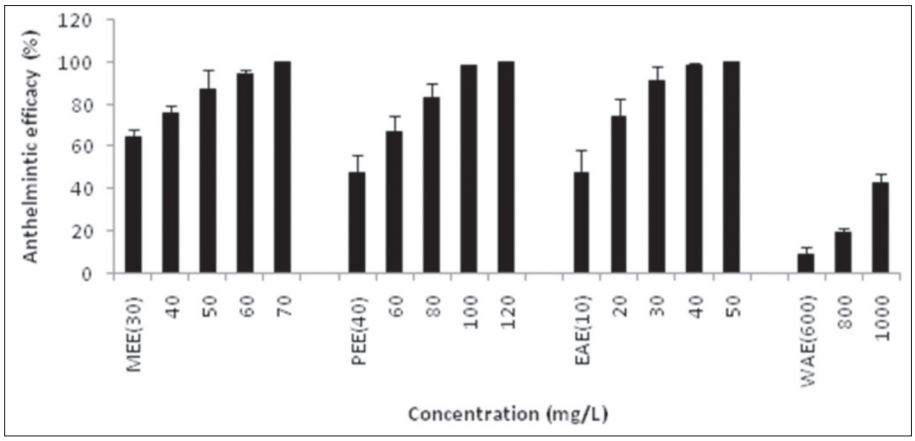


Fig 1. Anthelmintic activity of the different extracts of *Evodia rutaecarpa* against *Gyrodactylus kobayashii* after the exposure for 48 h. MEE, methanol extract; EAE, ethyl acetate extract; PEE, petroleum ether extract; WAE, water extract

Fig 2. Anthelmintic activity of the different extracts of *Cnidium monnieri* against *Gyrodactylus kobayashii* after the exposure for 48 h. MEE, methanol extract; EAE, ethyl acetate extract; PEE, petroleum ether extract; WAE, water extract



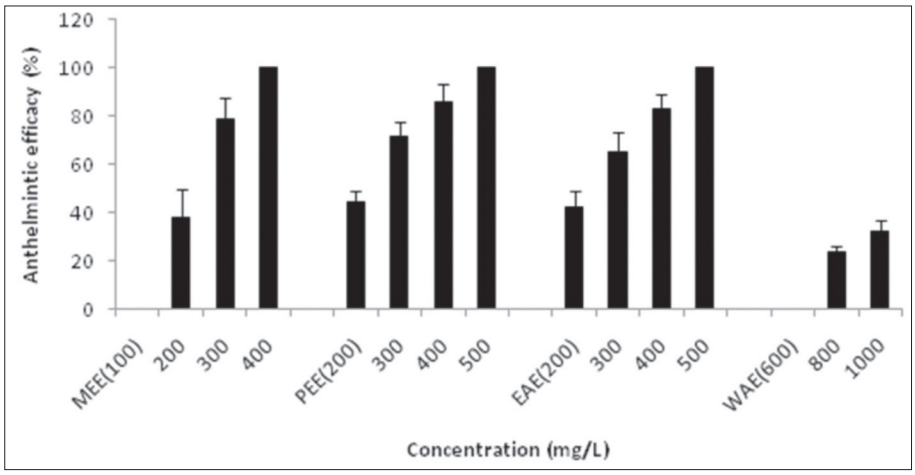


Fig 3. Anthelmintic activity of the different extracts of *Sophora flavescens* against *Gyrodactylus kobayashii* after the exposure for 48 h. MEE, methanol extract; EAE, ethyl acetate extract; PEE, petroleum ether extract; WAE, water extract

the extracts of *S. flavescens* showed effective anthelmintic efficacy against *G. kobayashii* after 48 h of exposure, but at high concentrations, with EC_{50} values of 234.6 mg/L (methanol), 224.4 mg/L (petroleum ether) and 238.6 mg/L (ethyl acetate).

Acute Toxicity of the Tested Extracts in Goldfish

The results of the acute toxicity assay of the extracts with higher anthelmintic efficacy at the low concentrations (methanol, petroleum ether, and ethyl acetate extracts of *E. rutaecarpa* and *C. monnieri*) are summarized in *Table 3*. After 48 h of exposure, the LC_{50} values of the extracts of *E. rutaecarpa* were 759.3 (methanol), 771.9 (petroleum ether) and 235.9 (ethyl acetate) mg/L, which is 18.6, 10.7 and 9.9 times higher than the corresponding EC_{50}, respectively. The methanol, petroleum ether, and ethyl acetate extracts of *C. monnieri* had LC_{50} values of 214.1, 405.3 and 350.0 mg/L, which is 9.1-, 9.1- and 31.8 times higher than the corresponding EC_{50}, respectively. The water extracts of *E. rutaecarpa* and *C. monnieri* exhibited very weak toxicity to goldfish, and killed only two and

Table 2. *Anthelmintic efficacy (EC_{50} and EC_{90}) of different extracts from Evodia rutaecarpa, Cnidium monnieri and Sophora flavescens against Gyrodactylus kobayashii after 48 h of exposure*

Plants	Extraction Solvent	EC_{50} (mg/L)	95% CI	EC_{90} (mg/L)	95% CI	P value
Evodia rutaecarpa	Methanol	40.9	0-56.0	91.2	76.3-131.1	0.06
	Petroleum ether	71.9	66.7-76.2	108.3	102.8-115.7	0.23
	Ethyl acetate	24.0	20.8-26.9	50.3	46.0-56.0	0.38
Cnidium monnieri	Methanol	23.6	15.8-28.4	51.0	47.6-55.8	0.46
	Petroleum ether	44.6	37.8-49.8	84.0	78.5-91.4	0.25
	Ethyl acetate	11.1	7.7-13.5	28.3	25.82-31.69	0.94
Sophora flavescens	Methanol	234.6	161.5-303.0	319.8	266.0-543.6	0.07
	Petroleum ether	224.4	0-292.8	400.0	328.1-714.7	0.11
	Ethyl acetate	238.6	0-315.3	416.4	335.9-914.6	0.06

EC_{50}, effective concentration with 50% anthelmintic efficacy; EC_{90}, effective concentration with 90% anthelmintic efficacy; 95% CI, 95% confidence interval

Table 3. *Acute toxicity for goldfish exposed to different extracts from Evodia rutaecarpa and Cnidium monnieri after 48 h of exposure*

Plants	Extraction Solvent	LC_{50} (mg/L)	95% CI	LC_{90} (mg/L)	95% CI	TI (LC_{50}/EC_{50})	P value
Evodia rutaecarpa	Methanol	759.3	713.5-806.6	854.7	807.3-975.2	18.6	0.45
	Petroleum ether	771.9	719.6-826.5	892.8	835.5-1039.1	10.7	0.32
	Ethyl acetate	235.9	172.6-298.8	385.9	317.2-579.7	9.9	0.70
	Water	>1000.0	-	-	-	-	-
Cnidium monnieri	Methanol	214.1	186.8-240.2	274.6	246.7-344.5	9.1	0.70
	Petroleum ether	405.3	332.0-462.8	552.9	488.5-735.8	9.1	0.30
	Ethyl acetate	350.0	301.5-398.5	453.6	403.7-579.8	31.8	0.59
	Water	>1000.0	-	-	-	-	-

LC_{50}, 50% lethal concentration; LC_{90}, 90% lethal concentration; 95% CI, 95% confidence interval; TI, therapeutic index; –, not calculated

one goldfish, respectively, at the concentration of 1000.0 mg/L.

DISCUSSION

Diseases caused by the common ectoparasites, *Gyrodactylus*, can result in high mortality and huge financial losses in commercially farmed fish [6,17]. Additionally, prolonged and frequent use of common chemicals such as formaldehyde, rotenone, and praziquantel results in increasing drug resistance and adverse effects on the environment [8,10]. Therefore, there is an urgent need for effective strategies to control *Gyrodactylus* infections. In our study, the ethyl acetate extract of *C. monnieri* was the most efficient, having the lowest EC_{50} and EC_{90} values (11.0 and 28.3 mg/L, respectively) after 48 h of exposure, and it had 100% anthelmintic efficacy against *G. kobayashii* at 50.0 mg/L. The dried fruit of *C. monnieri* (L.) Cuss., called "Shechuangzi" in Chinese, is a commonly used traditional Chinese medicine with an impressive range of health benefits [18]. Previous research has found that the ethanol extract had better anthelmintic efficacy against *Dactylogyrus intermedius* (*D. intermedius*) in goldfish, and had the minimal effective concentration compared to the chloroform, petroleum ether, water, and acetone extracts of *C. monnieri*. This extract had 100% anthelmintic efficacy at 70.0 mg/L after 48 h of exposure. The active compounds of the ethanol extract against *D. intermedius* were identified as osthol and isopimpinellin [19,20]. In the present study, the ethyl acetate extract of *C. monnieri* was the most effective against *G. kobayashii* in goldfish at the minimal concentration, showing 100% anthelmintic efficacy at 50.0 mg/L, which was far lower than the concentration of ethanol extract with 100% anthelmintic efficacy (100.0 mg/L). Differences in anthelmintic efficacy of the same extracts may be due to the differences in the evaluation methods. In previous research, anthelmintic efficacy was calculated by comparison with the control group. In our paper, the number of *G. kobayashii* was recorded before and after exposure, and anthelmintic efficacy was calculated based on the changes in intensity of *Gyrodactylus* in goldfish. Besides, the difference in the main parasitic sites may be another cause. The main parasitic site of *Dactylogyrus* sp. is the gill, which may provide partial protection for this parasite by reducing the exposure to extracts. Nevertheless, *Gyrodactylus* sp. is mainly parasitic on the fins or the skin, which directly exposes this parasite to the extracts.

"Wuzhuyu", derived from the dried fruit of *E. rutaecarpa* (Juss.) Benth., is well described in the Chinese medical matter

and the Chinese pharmacopoeia, and it is recommended for the treatment of dizziness, headache, emesis, diarrhea and other symptoms [21,22]. Pharmacological studies indicate that the bioactive constituents of *E. rutaecarpa* have many properties, including anti-inflammatory, antihypertensive, antinociceptive, antimicrobial and anthelmintic activities [23,24]. For example, Perrett and Whitfield [25] reported that atanine (3-Dimethylallyl-4-methoxy-2-quinolone), an alkaloid isolated from the hexane extract of *E. rutaecarpa* caused 100% immobility of the cercarial and miracidial larvae of *Schistosoma mansoni* at 100.0 mg/L, after 7 min of exposure. Liu et al.[26] have shown that ethanol extracts of *E. rutaecarpa* are effective at killing intestinal nematode parasites of pigs and leeches *in vitro*. Also, the n-hexane extract of *E. rutaecarpa* has been demonstrated to have strong antifeedant activity against the grain storage insects *Sitophilus zeamais* and *Tribolium castaneum* [26]. Moreover, two alkaloids isolated from the methanol extract of *E. rutaecarpa*, evodiamine and rutaecarpine, have shown insecticidal activity against fruit flies (*Drosophila melanogaster*), with LC_{50} values of 3.9 and 3.6 mg per adult, respectively [27]. In the present study, the ethyl acetate extract of *E. rutaecarpa* showed good anthelmintic efficacy, with EC_{50} and EC_{90} values of 24.0 and 50.3 mg/L, after 48 h of exposure. Although the active compounds against *G. kobayashii* that are contained in the ethyl acetate extract have not been identified, some of the compounds described above are thought to jointly or independently control *G. kobayashii* infection.

The therapeutic index (TI), aparameter for the quantitative relationship between efficacy and safety, is used to evaluate the potential application of the extracts in aquaculture [28]. TI is calculated as the radio of LC_{50} to EC_{50}, and higher TI values reflect safer effects. In our study, we found that the ethyl acetate extract of *C. monnieri* had the highest TI value of 31.8, and the lowest EC_{50}, which indicated this extract was safest to goldfish among all extracts and had the potential to be used in aquaculture. Additionally, the methanol extract had a higher TI value of 18.6, but the EC_{50} was also higher compared with the ethyl acetate extract for *E. rutaecarpa*. The 48-h LC_{50} values of the remaining extracts of *C. monnieri* and *E. rutaecarpa* were about 10-fold higher than the corresponding EC_{50} values, which reflects low toxicity to goldfish. The bioactive ingredients that play a major role in anthelmintic activity are necessary for further bioassay-guided isolation and identification. In addition, field trials need to be performed before the use of extracts from *C. monnieri* and *E. rutaecarpa* in aquaculture.

Among the 14 Chinese medicinal herbs screened, the extracts of *C. monnieri* and *E. rutaecarpa* had the highest anthelmintic efficacy against *G. kobayashii* in goldfish. The ethyl acetate extract of *C. monnieri* was the most effective and the safest of all the extracts tested. *C. monnieri* and *E. rutaecarpa* may become novel therapeutic agents against *G. kobayashii* infection.

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Competing Interests

The authors declare they have no competing interests.

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Research Article

Higenamine Decreased Oxidative Kidney Damage Induced By Ischemia Reperfusion in Rats

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Abstract

The aim of this research is to determine protective effects of higenamine on kidney tissue injury caused by ischemia reperfusion. In this study, 24 Sprague Dawley female rats were divided into 3 groups. The groups were designed as follows; control, ischemia reperfusion, and ischemia reperfusion + higenamine. Some oxidant, antioxidant and inflammatory parameters were evaluated in kidney tissues at the end of the experimental procedure. It was confirmed that the oxidant and inflammatory parameters of kidney tissue increased and antioxidant parameters decreased in ischemia reperfusion group compared to control group. Antioxidant parameters increased while oxidant and inflammatory parameters decreased in the ischemia reperfusion + higenamine group compared to ischemia reperfusion group. These results have demonstrated that higenamine administration as single dose is effective against oxidative kidney damage originating from ischemia reperfusion.

Keywords: *Ischemia reperfusion, Higenamine, Kidney, Oxidative stress, Inflammation, Rat*

Higenamin Ratlarda İskemi Reperfüzyonunun Neden Olduğu Oksidatif Böbrek Hasarını Azaltır

Öz

Bu araştırmanın amacı, higenaminin, iskemi reperfüzyonunun neden olduğu böbrek dokusu hasarı üzerine koruyucu etkilerini belirlemektir. Bu araştırmada 24 adet Sprague Dawley dişi sıçan üç gruba ayrıldı. Bu çalışmanın grupları aşağıdaki şekilde tasarlanmıştır; kontrol, iskemi reperfüzyon ve iskemi reperfüzyon + higenamin grupları. Deney sonunda elde edilen böbrek dokularındaki bazı oksidan, antioksidan ve inflamatuvar parametreler değerlendirildi. İskemi reperfüzyon grubu kontrol grubu ile kıyaslandığında, böbrek dokusundaki oksidan ve inflamatuvar parametrelerin arttığı fakat antioksidan parametrelerin azaldığı belirlendi. Tedavi grubu (iskemi reperfüzyon + higenamin) yalnızca iskemi reperfüzyon grubu ile kıyaslandığında antioksidan parametreler artarken, oksidan ve inflamatuvar parametreler azaldı. Bu sonuçlar, tek doz higenamin uygulamasının, iskemi reperfüzyon kaynaklı oksidatif böbrek hasarına karşı etkili olduğunu göstermiştir.

Anahtar sözcükler: *İskemi reperfüzyon, Higenamin, Böbrek, Oksidatif stres, İnflamasyon, Sıçan*

INTRODUCTION

Decreasing the blood flow, reperfusion and systemic inflammatory response may lead to kidney ischemia reperfusion (I/R) injury [1]. Acute kidney injury (AKI) is related to a severe mortality, great economic, and social burdens, particularly in critically ill cases [2-4]. In the long term, AKI may cause chronic kidney disease and end-stage kidney disease [5]. Reactive oxygen species (ROS), are related to the early phase of inflammation, necrosis and apoptosis in kidney I/R injury [6]. It has been suggested that increased ROS production during kidney I/R is one of the most important reasons of kidney damage with extensive interstitial edema, tubular flattening with brush border

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microvilli loss, tubular dilatation, brush border shedding, casts and obstruction [7,8]. Elevated malondialdehyde (MDA) levels were shown due to I/R injury and activities of antioxidants (ROS scavenger) such as catalase (CAT) and superoxide dismutase (SOD) were determined to be essential to prevent the toxic effects of MDA [9]. MDA is a lipid peroxidation product and it is used to evaluate the oxidative stress levels in *in vitro* and *in vivo* conditions [10]. Linas et al.[11] indicated that kidney I/R injury was aggravated by activated neutrophils. Neutrophil activation is related with myeloperoxidase (MPO). Furthermore, proinflammatory cytokine and ROS production is associated with active neutrophils [12]. I/R injury aggregates inflammatory cells, releases inflammatory factors (TNF-α, IL-8, and IL-6, etc.) and increases adhesion molecules [13,14]. Up to day, there has been no efficient therapy against kidney I/R injury [15,16].

Higenamine (Hig) (1-[(4-hydroxyphenyl)methyl]-1,2,3,4-tetrahydroisoquinoline-6,7-diol) is an alkaloid and was first derived from Aconitum [17]. As a source for Hig, *Aconicum japonicum* Thunb has been used for collapse, tumor, bronchial asthma, rheumatic fever, edema, painful joint, and syncope treatment for centuries in China and Japan [18]. In 1976, aconite root was used for the first isolation of Hig [19]. Hig has pharmacological features including immunomodulatory, antiapopitotic, anti-inflammatory and antithrombotic effects [17]. This research was planned to detect the protective effect of Hig against kidney oxidative damage induced by I/R.

MATERIAL and METHODS

This study was initiated with approval (2019-69) of Atatürk University Experimental Animals Local Ethics Committee. Experimental phase of the present research was performed at Atatürk University Experimental Animal Research and Application Center and the animals were supplied from the same place. Animals were kept in standard cages in laboratory environment provided with humidity, 20-22°C temperature and 12 h light/dark cycle control. They were fed with standard pellet feed and water. They were fasted 12 h before the experiment to prevent anesthesia complications.

Groups and Ischemia Reperfusion Model

All procedures were performed under anesthesia of 10 mg/kg i.p xylazine hydrochloride (Rompun®, Bayer, Istanbul) and 60 mg/kg i.p ketamine (Ketalar®, Pfizer, Istanbul). The number of animals and there should be at least 8 animals in each group if the animals were divided into 3 groups were determined by 5% deviation, type 1 error (α) 0.05 and type 2 error (β) (Power = 0.80) power analysis. Three groups each containing 8 Sprague Dawley female rats (240±10 g) were set. Control group; the back region was shaved, cleaned and opened with an incision under anesthesia and then closed without I/R model or a medication. I/R group; the incision area was cleaned with povidone iodine, opened with an incision under anesthesia and then, bilateral kidney arteria and veins were blocked with an atraumatic microvascular clamp for 1 h. In reperfusion period, blood circulation was allowed for 24 h by opening the clamps. Incision closed with silk 3/0 suture. I/R + Hig group; Hig was administered to rats intraperitoneally at a dose of 10 mg/kg 30 min before reperfusion. Later as described in I/R group, the I/R model was created. At the end of the experiment, the right kidneys of all decapitated rats were collected with capsules. Finally, when the experiment ended, the kidney tissues were washed and kept frozen until the biochemical analysis. Hig was purchased from Sigma Aldrich (Missouri, USA).

Analysis of Biochemical Parameters

The kidney samples (right kidney samples with capsules, each sample 100 mg) were homogenized with phosphate buffer (2 mL). The homogenized kidney tissues were centrifuged at 5000 rpm at +4°C for 20 min; and the supernatants obtained in this way were transferred to microcentrifuge tubes. Kidney tissue samples were processed for MDA assay to determine lipid peroxidation status as described by Ohkawa et al.[20]. The results were given in µmol/g protein. SOD activity was measured as defined by Sun et al.[21]. The results were presented in U/mg protein. We also quantified kidney injuries by measuring tissue MPO activity, using a protocol developed by Bradley et al.[22]. The results of MPO activity in tissue samples were presented in U/g protein. The total antioxidant status (TAS) value was evaluated with a commercially available kit (Rel Assay Diagnostics). Total oxidant status (TOS) measurement was done with a commercially available kit (Rel Assay Diagnostics). TAS and TOS results were presented as nmol/L. TOS to TAS ratio was accepted as the oxidative stress index (OSI). OSI level was detected as follows: OSI = [(TOS, µmol H_2O_2 equivalent/L)/(TAS, mmol Trolox equivalent/L) × 10]. OSI has been proposed to be better in demonstrating the oxidative state more precisely compared to TOS value [23].

Statistical Analysis

Statistical analysis was performed using IBM SPSS Package Program (version 22.0). Experimental results were reported as mean ± standard deviation. The normality assumption was confirmed by the Kolmogorov Smirnov test. One-way ANOVA was used to compare the experimental groups with the control. Multiple comparisons were made using the Post hoc Tukey test.

RESULTS

While TAS value decreased significantly, TOS and OSI levels increased in I/R group compared to control group. TOS and OSI values decreased but TAS level increased in I/R + Hig group compared to group I/R (*Table 1*, $P<0.05$).

Table 1. *TAS (mmol/L), TOS (μmol/L) and OSI values of control, I/R and I/R+Hig groups*

Groups/Parameters	Control	I/R	I/R+Hig
TAS (mmol/L)	2.60±0.15	1.39±0.15[a]	2.53±0.15[b]
TOS (μmol /L)	7.09±0.53	10.05±0.93[a]	7.50±0.68[b]
OSI	0.27±.02	0.72±0.10[a]	0.29±0.03[b]

The results are presented as mean ± SD (n=8). [a] P<0.05 versus to control group, [b] P<0.05 versus to I/R group

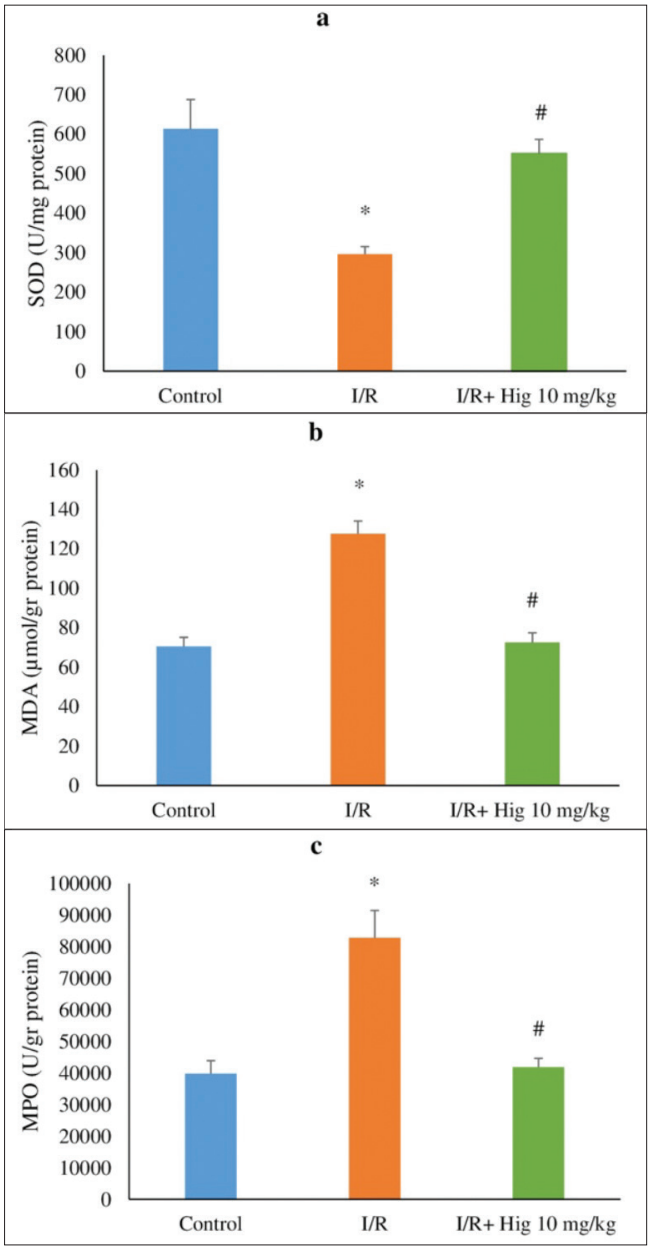

Fig 1. Biochemical parameters in kidney tissue. **a-** SOD levels, **b-** MDA levels and **c-** MPO activity. Data are expressed as mean ± SD; * P<0.05 versus to control group, # P<0.05 versus to I/R groups

Superoxide dismutase enzyme activity increased in I/R + Hig group compared to I/R group. The antioxidant and anti-inflammatory properties and protective effects of Hig against I/R-induced kidney injury have been demonstrated by biochemical results (as shown in *Fig. 1-a)*. However, MPO activity *(Fig. 1-b)*, MDA *(Fig. 1-c)*, TNF-α *(Fig. 2-a)* and IL-1β *(Fig. 2-b)* levels increased in I/R group compared to control group and decreased in I/R + Hig group compared to IR group (P<0.05).

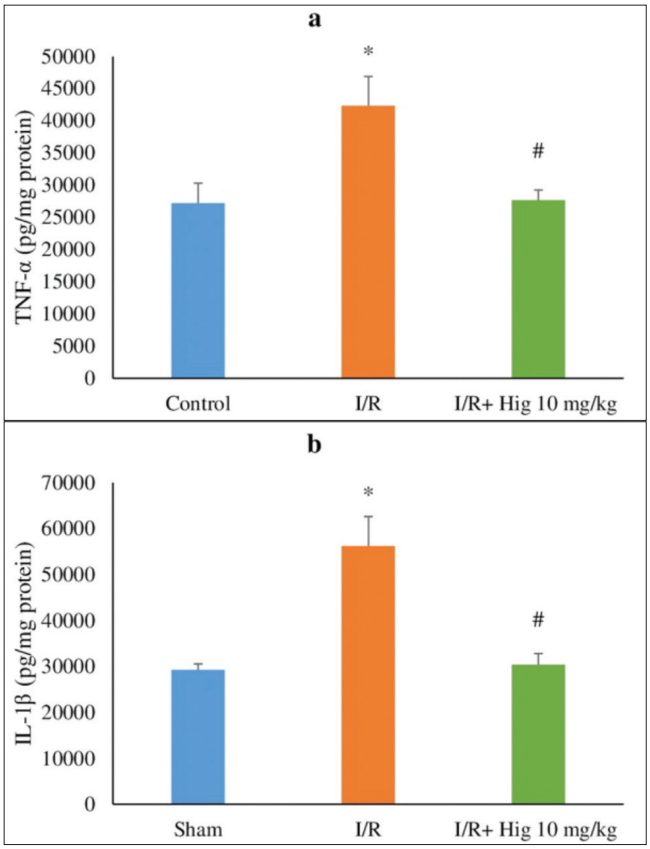

Fig 2. Inflammatory markers in kidney. **a-** TNF-α levels and **b-** IL-1β levels. Data are expressed as mean ± SD (n=8). * P<0.05 versus to control group, # P<0.05 versus to I/R groups

DISCUSSION

Acute kidney injury, especially in developing countries, is related to mortality and morbidity [24]. AKI usually occurs due to I/R injury [25]. Kidney I/R injury is a major reason for AKI with various origins such as kidney transplantation, shock and low cardiac output [26]. Kidney injury following kidney transplantation may also lead to kidney I/R injury [27]. In the reperfusion phase, oxygen derived free radicals occur [28]. Tissue injury induced by I/R is based on oxidative stress and this condition is supported with a strong body of evidence [29]. To remove toxic ROS, cells have several

natural defense systems, including SOD enzyme. Increased ROS that is generated during I/R may cause endogenous antioxidant depletion [30]. The protecting enzymes (SOD, CAT) perform against the devastating actions of ROS and these molecules comprise TAS. TAS measurement provides the evaluating of all antioxidant levels in a biological sample [31]. TOS to TAS ratio is confirmed as OSI, which is an indication of oxidative stress [32]. Reaction between ROS and lipids causes lipid peroxidation in biological membranes during kidney I/R injury [33] and ultimately, enzymatic actions like ion pump activity (plays role on inhibition of DNA transcription and repair) is affected. If an uncontrolled lipid peroxidation continues, it may end with cell death [34,35]. MDA, bioproducts of lipid peroxidation, indicates oxidative stress. CAT and SOD indirectly show free radical generation ability. These are positive and negative markers for oxidative stress level [36]. MDA was clearly increased in a kidney I/R model in rats [37].

The inflammatory response is another important part of the pathophysiology implicated in kidney I/R injury [38]. Some proinflammatory cytokines such as IL-2, IL-6, TNF-α and IL-1β are released during kidney I/R injury [39,40]. TNF-α takes an important part in the beginning and continuation of the inflammatory response. Further, TNF-α could lead to endothelial damage, apoptosis and even kidney failure [41]. IL-1 is a proinflammatory cytokine involved in several inflammatory processes [42]. When the inflammatory response is regulated at the early stage effectively, this presents a vital step for prevention and treatment of kidney injury [43,44].

There are several studies showing the antioxidant and anti-inflammatory properties of Hig that support the results of this study. In the present study, reduction of IL-1β and TNF-α levels in kidney I/R model in rats by Hig, suggesting that Hig decreased IR-induced kidney injury. In a rat model of cerebral I/R, Hig improved functional state of nerves and significantly stopped the increase in TNF-α, IL1, and IL-6 levels while decreasing the axonal nerve degeneration [45]. Induction of ROS and MDA production, and SOD activity inhibition arising from neuronal cell injury triggered by oxygen-glucose deprivation/reperfusion were attenuated by Hig [46]. Hig increased antioxidant level and reduced MDA, TNF-α and IL-1β levels in a collagen-induced arthritis study [47]. According to published reports, Hig possesses a variety of pharmacological properties, including dilatation of blood vessels and bronchi, immunomodulatory, anti-inflammatory, antiapoptocic and antioxidation features [18]. In parallel with these studies, in the present study anti-oxidant and antiinflammatory properties of Hig have been shown in kidney I/R model in rats. In I/R group, TAS and SOD values decreased while TOS, OSI, MDA, MPO, TNF-α, and IL-1β levels increased compared to control group and Hig treatment reversed these levels.

Oxidative stress in kidney tissue was assessed to determine the possible mechanisms of the protective effect of Hig against I/R-induced kidney injury and it was observed that oxidative stress decreased with Hig. To make effective changes in the clinical management of I/R, the pathogenesis of I/R-induced organ damage should be better understood for the development of therapeutic strategies. Clearly observed in I/R studies is that suppression of inflammation and oxidative stress can provide significant contributions to I/R treatment. In the present study, inflammation and oxidative stress pathways are suppressed by Hig and this promises hope in the treatment of I/R.

Hig provides a protection against I/R-induced kidney injury with its antioxidant and anti-inflammatory properties. We have indicated that treatment with Hig reduces kidney injury in experimental animals exposed to I/R model. Moreover, further researches are necessarry to explain the other protective mechanisms in I/R-induced kidney tissue injury.

Conflict of Interest Statement

None.

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Research Article

Generation of Ectodysplasin A *(eda)*-targeted Knockout Zebrafish Via the CRISPR/Cas9 System

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Abstract

Ectodysplasin A (EDA) plays a vital role in the development of skin appendages, especially in fish scales. Zebrafish model with the mutation of *eda* was found using CRISPR/Cas9 system. CRISPR/Cas9 nucleases targeting to two loci in exon 4 of *eda*, were constructed and injected into zebrafish embryos, respectively. CRISPR-Cas9 mediated mutation frequency toward eda exon 4 was approximately 16%, which was relatively low compared with that of other genes in zebrafish. Five *eda* mutant types were obtained in F0 generation including a deletion of 5 bp, 6 bp, 8 bp and 87 bp, and an insertion of 11 bp around the targeted site respectively, and all of which happened just in one allele. But the scales of all F0 founders were normal compared with their wild counterparts. In the F1 generation, five scale loss mutants with few scales covered were achieved that were all caused by bi-allelic 11-bp insertion in *eda*. The insertion results in frameshift mutation of *eda* and leads to loss of expression and function inactivation of EDA as determined by western blotting. This provides a good model for elucidating the function of EDA in the development of fish scales.

Keywords: *Gene knockout, eda, Zebrafish mutant, Scale loss*

CRISPR/Cas9 Sistemi Kullanılarak Ectodysplasin A *(eda)* İfade Etmeyen Zebra Balığı Üretimi

Öz

Ektodisplasin A (EDA) deri uzantılarının gelişiminde, özellikle balık pullarında, hayati bir rol oynar. CRISPR/Cas9 sistemi kullanılarak *eda* mutasyonlu zebra balığı modeli bulundu. *Eda*'nın ekson 4'ünde iki lokusu hedefleyen CRISPR/Cas9 nükleazları oluşturuldu ve zebra balığı embriyolarına enjekte edildi. *Eda* ekson 4'e yönelik CRISPR-Cas9 aracılı mutasyon sıklığı yaklaşık %16 idi; bu, zebra balığındaki diğer genlerinkine kıyasla nispeten düşüktü. F0 jenerasyonunda, hedeflenen bölge etrafında sırasıyla 5 bp, 6 bp, 8 bp ve 87 bp'lik bir silme ve 11 bp'lik bir ekleme dahil olmak üzere beş *eda* mutant türü elde edildi ve bunların hepsi sadece bir allelde gerçekleşti. Ancak tüm F0 üyelerinin pulları yabani akranlarına kıyasla normaldi. F1 jenerasyonunda, tamamı *eda*'ya bi-allelik 11-bp ilavesinden kaynaklanan az sayıda pulla çevrili beş adet pulsuz mutant elde edildi. Ekleme, *eda*'nın çerçeve kayması mutasyonuna neden olur ve Western Blot tekniği ile belirlendiği üzere EDA'nın ekspresyonunda azalma ve fonksiyon inaktivasyonuna yol açar. Bu çalışma, balık pullarının gelişiminde EDA'nın işlevini açıklamak için iyi bir model oluşturmaktadır.

Anahtar sözcükler: *Gen nakavt, eda, Zebra balığı mutantı, Pul kaybı*

INTRODUCTION

In the last decade, the emergence of three major genome-engineering technologies have provided efficient support for site-specific modification of genes in various species [1]. The core principle of engineered zinc-finger nuclease (ZFN), transcription activator like effector nuclease (TALEN), and clustered regularly interspaced short palindromic

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repeats (CRISPR)/CRISPR-associated Cas9 is recognizing and introducing double-strand breaks (DSBs) in targeted region of the genome precisely. In comparison, CRISPR/Cas9 systems make this process much more feasible and efficient.

Ectodysplasin A (EDA) belongs to TNF family which involved in the development of various structures derived from the ectoderm, including hair, teeth, sweat glands, feathers, armor plates, and scales. The EDA protein contains four transmembrane region (TM) domains, a furin consensus cleavage site, a collagen like domain (CL), and a tumor necrosis factor domain (TNF) [2]. In humans, X-linked hypohidrotic ecto-dermal dysplasia (XLHED) is a rare, inherited disorder that has been observed globally [3]. The phenotypic characteristics associated with this disorder include sparse hair, abnormal or missing teeth, and inability to sweat as a result of absent sweat glands. Several different inheritance patterns have been observed in relation to XLHED. The majority of these cases were caused by mutations in the *eda*1 gene [4]. In mice, Srivastava et al.[5] found that the tabby phenotype (characteristic hair defects, tooth abnormalities, and eccrine sweat gland morphology) was caused by a mutation in *eda*. *eda* mutants in above studies associated the development defects of skin attachment and accumulation with the TNF regions. In zebrafish, Harris et al.[6] screened a similar scale loss mutant caused by a mutation location in the TNF domain of EDA, demonstrating that TNF domain played a vital role in the development of skin appendages. Nevertheless, several in-frame deletions have been observed in HED patients and Schizothoracinae fishes [7-10], indicating that the collagen-like domain also harbored other important functions which are still unknown.

Therefore, in order to determine the function of other domains in the EDA protein except TNF, we employed CRISPR/Cas9 systems to induce mutation in the *eda* of zebrafish and achieved the scale loss mutants. It appears that it will provide a good model for researching the genetic mechanisms of skin appendage development, drug screening, and gene function identification.

MATERIAL and METHODS

Targeted Sites Design

In order to determine the role of EDA CL domain in fish scale development, we designed a CRISPR/Cas9 system to knockout *eda* in the zebrafish genome. According to the sequence alignment results, the CL domain is coded by exon 4 in zebrafish EDA. Therefore, two target sites were selected *(Fig. 1)* at the *eda* exon 4 locus based on online ZIFIT software *(http://zifit.partners.org/ZiFiT/)*. Accordingly two small guide RNAs targeting to the screened sites were designed namely gRNA-1(TTAGGCAAGAAAGGGCCCCCTGG) and gRNA -2(AGCAACGCCATGGGTCCCTCTGG)

Plasmid Construction and RNA Synthesis

According to Hwang et al.[11], the plasmids required to practice CRISPR-Cas/RNA-Guided Nuclease (RGN) technology in zebrafish include Cas9 expression vector and guide RNA expression vector. The Cas9 expression plasmid MLM3613 and the guide RNA expression vector were a gift from Keith Joung (Addgene plasmid # 42251 and # 42250; http://n2t.net/addgene: 42251, 42250; RRID: Addgene_42251 and 42250). The methods used to facilitate plasmid construction and RNA synthesis are as follows [11]. In brief, the guide RNAs gRNA -1 and gRNA -2 (comprising a 20-bp target sequence complementary to the genomic target adjacent to a protospacer adjacent motif (PAM) with a sequence of NGG) toward the selected targeting sites were cloned into the pDR274 vector cut by *Bsa*I. The obtained plasmid were named as pDR274-eda1 and pDR274-eda2 which encoding sgRNA-1 and sgRNA-2, respectively. Cas9 mRNA was transcribed using *Pme*I-digested pMLM3613 as template by mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). sgRNA-1 and sgRNA-2 were transcribed using *Dra*I-digested pDR274-*eda*1 and pDR274-*eda*2 as templates by the MAXIscript T7 kit (Life Technologies).

Experimental Animals

According to the standard of zebrafish breeding, adult zebrafish were bred at 28°C in circulating water on a 14 h light cycle followed by a 10 h dark cycle and fed twice daily. The injected eggs were incubated at 28.5°C in an embryo medium, to the larvae out of the membrane and the yolk sac disappear, then the larvae were fed with yolk as opening bait twice-daily. All animal experiments were approved by the Animal Care and Use Committees of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences and the Agriculture Department of Qinghai Province, China.

Microinjection and Activity Determination

The mixtures containing 50 pg of sgRNA-1 and 250 pg of Cas9 mRNA, or 50 pg sgRNA-2 and 250 pg Cas9 mRNA, were injected directly into 70 single-cell-stage embryos as described by Hwang et al.[11], respectively. Aind the embryos were derived from the Tübingen wild-type cross. 24 h after injection, we collected randomly 20 embryos which were lysed as template of PCR to detect whether mutations were introduced near the PAM site, the remaining embryos were kept until 3 months of age. The remaining 50 fish were sedated in 0.016% solution of Tricaine (Sigma) until the startle response ceased and buoyancy equilibrium was lost, after which fin clips were obtained using a clean eye scissors. Post-op recovery tanks contained dilute methylene blue as anti-infection treatment. Genomic DNA was extracted using Genome extraction kit (B518221, Sangon Biotech). A pair of primers (F: TTGTTTTGCTTCTCATCAGTTG, R: TTTGCTCTGCTGCTTCACTC) around the PAM sites were

designed and used to detect the mutation events. The length of products using the primers and with wild zebrafish genomic DNA as template is 357 bp. The following procedure was used to amplify the targeted sequences: pre-denaturation was performed at 94°C for 5 min; then 32 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec; and finally extended at 72°C for 7 min using Veriti Thermal Cycler PCR system (Applied Biosystems, USA). Appropriate PCR products with length between the range of 250 bp and 450 bp were sequenced directly, and those showing multi-peaks set close to the PAM site were cloned into pMD18-T, then sent to further sequence and analysis. The zebrafish with the sequence differed from that of their wild type (such as substitution or transversion of single nucleotide, and insertion or deletion of short nucleotides) around the PAM sites were named as F0 founder.

F1 Mutant Detection and Observation

The day before breeding, using partitions to separate the breeding male and female F0 founders at 10-hour dark cycle. The next day removed partitions and let the parents fish mated randomly. Resulting embryos were collected and grown to 45 days of age, and then PCR was performed to identify gene-targeted modifying events. The F1 mutants were further stained via Alizarin red to observe the patterns of scale development as described by Harris et al.[6] with microscope (OLYMPUS SZ61).

Western Blotting

The whole bodies of the two samples (one EDA mutant and one wild-type fish) were frozen in liquid nitrogen and homogenized with electric homogenizer adding lysing RIPA buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA), respectively. After shaking for 1 h, the supernatant fluid containing the whole protein were obtained through centrifugation at 4°C (13000 rpm for 5 min). 20 μL of total protein were separated by 10% SDS-PAGE at 100V for 1.5-2 h, and then transfered to nitrocellulose membranes at 100V for 1-1.5 h. Membranes were blocked in 5% milk in TBST (TBS with 0.05% Tween) overnight at 4°C. The primary antibodies, rabbit polyclonal antibodies against human EDA (ab84311, Abcam) and against zebrafish β-actin (ab16039, Abcam), were diluted 1:5000 with antibody solution, and incubated with the blocked membrane in blocking buffer overnight at 4°C. Washing the membrane at least three times with TBST, 5 min each, and then incubating with the secondary antibody of Goat Anti-Rabbit HRP (ab205718, Abcam, 1:5000) in blocking buffer at room temperature for 1 h. Images were acquired using darkroom development techniques for chemiluminescence and ChemiDoc XRS+ (BIO-RAD).

RESULTS

Targeted Site Design and Activity Detection

In this work, we carried out a targeted disruption of the *eda* exon 4 loci in zebrafish using CRISPR-Cas9 system *(Fig. 1)* to further verify the function of EDA CL domain in fish scale development. Two designed small guide RNA, namely sgRNA-1 and sgRNA-2 were injected into 70 one-cell stage embryos, respectively. According to the sequencing results of target sites, eight individuals of F0 generation showed multi-peaks set close to the PAM sequence *(Fig. 2)* in the 50 adults after injection of sgRNA-1; CRISPR-Cas9-mediated mutation frequency within the *eda* exon 4 was approximately 16%. Nevertheless, multi-peaks sets were observed in the 70 embryos by injecting sgRNA-2. It was estimated that there was no activity of sgRNA-2 *in vivo*.

Mutation Detection and Typing

According to *Fig. 2*, deletion and insertion mutation types were introduced near the PAM site in founder 0 (F0). The screened five *eda* mutation types including a deletion of 6 bp (F0 1#, 2#), a deletion of 5 bp (F0 3#, 4#), a deletion of 87 bp (F0 5#), an insertion of 11 bp (F0 6#, 7#), and a

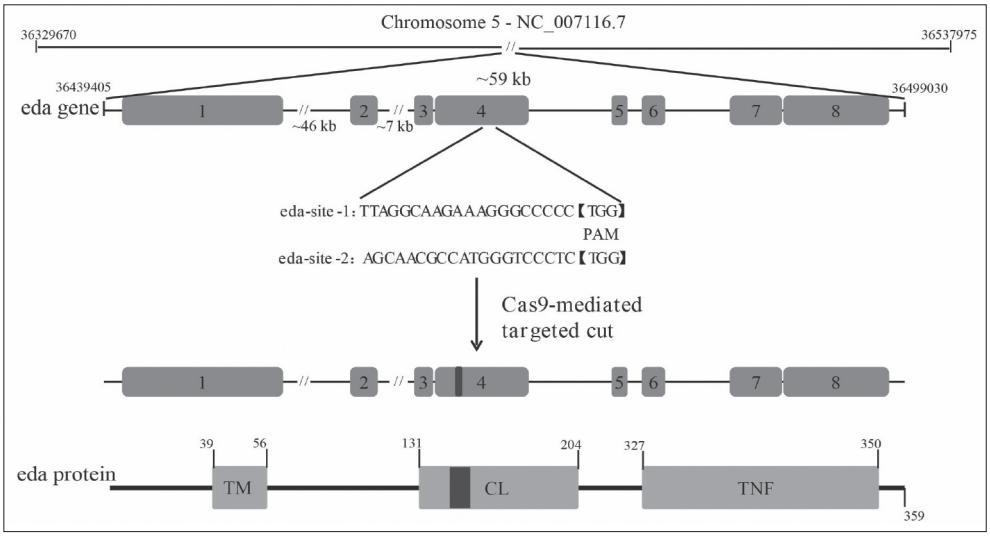

Fig 1. Engineered CRISPR-Cas systems and associated target Eda gene loci used in this study. The Eda gene has eight exons (gray box) in zebrafish. We designed two target sites including Eda-site-1 and Eda-site-2 at exon 4 on chromosome 5. The TGG of the target sequence is the protospacer adjacent motif (PAM). When sgRNA-1 and sgRNA-2 were injected into the single-cell stage embryos, the mutation (black bar) will be induced at the exon 4 locus. EDA protein are mainly transmembrane (TM), collagen-like (CL) and tumor necrosis factor (TNF) domains, and the CL domain are edited by exon 4

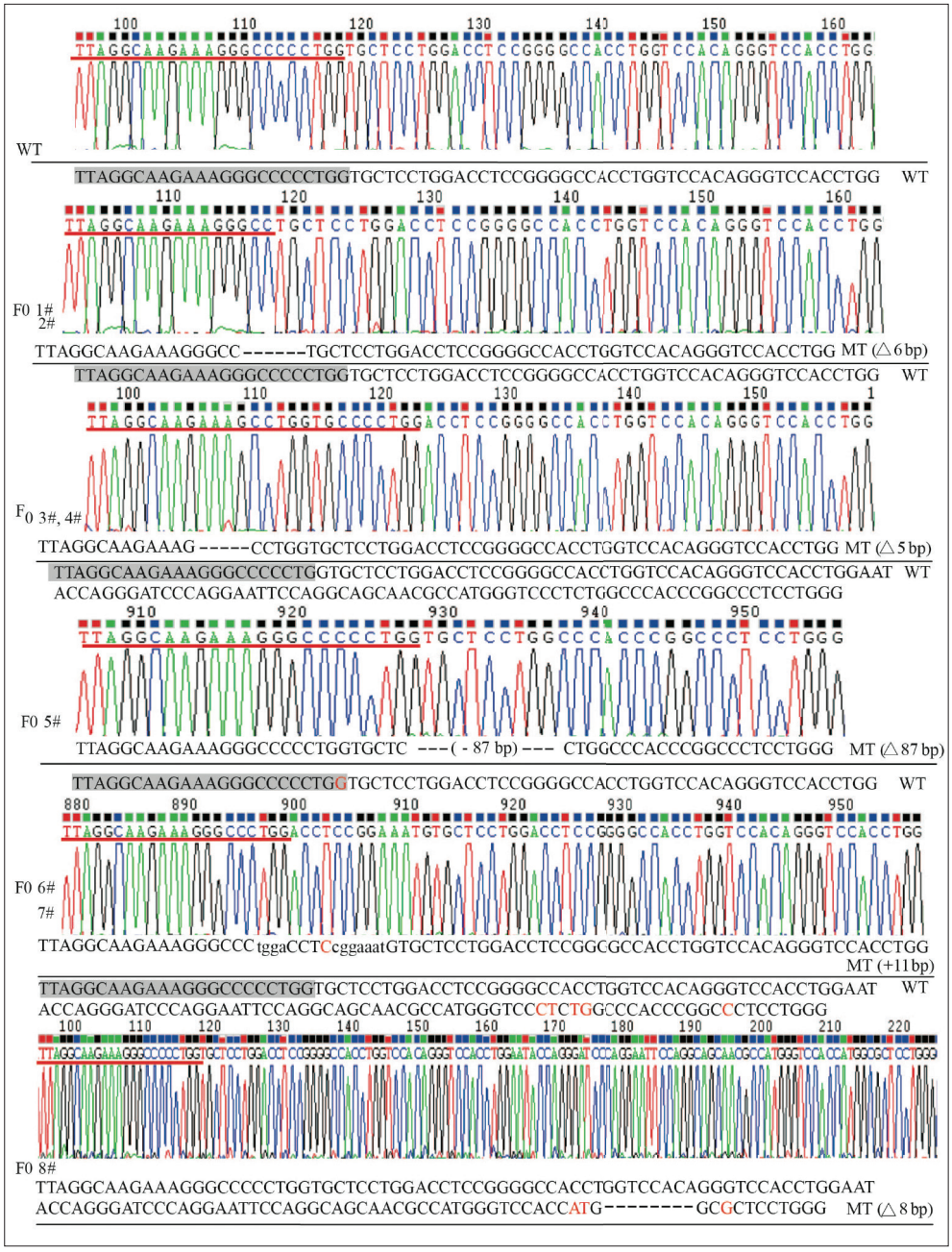

Fig 2. The PCR products detected in the founder 0 (F0) mutated zebrafish induced by injection of Cas9 enzyme together with sgRNA. Double-peaks and the sequences corresponding mutants were detected near the PAM site in the wild type (WT), 1#, 2#, 3#, 4#, 5#, 6#, 7#, and 8# mutants, and the results suggested that deletion and insertion mutations were induced close to the PAM site. The target sequence including PAM sites are marked in light gray and red line. Abbreviations: deletion sequences are indicated by "-----", insertion sequence is lowercase letters; Wild-type (WT), mutation-type (MT), deletion/insertion (△/+)

deletion of 8 bp (F0 8#). Among them, the deletion of 5 bp, a deletion of 6 bp, and the insertion of 11 bp were detected in two individuals, respectively. An ORF analysis indicated that the deletion of 5 bp, 8 bp and the deletion of 87 bp were in-frame mutations, while the deletion of 6 bp and the insertion of 11 bp were out-frame mutations. The scale phenotype of all F0 mutation types were normal by microscopic observation (OLYMPUS SZ61).

Scale-loss Mutants

In this research, the two male F0 founders (6# and 8#), were mated randomly with the six female F0 founders (1#, 2#, 3#, 4#, 5#, and 7#). Then we obtained 53 embryos and eventually 26 adult zebrafish after feeding for three months, the survival rate of 49% is half that of the wild-type zebrafish (90%). This might be due to inbreeding or the mutations leading to decrease in their survival ability, but it served to remind us to maintain stringent conditions in the mutant breeding program. The 26 living zebrafish were detected by microscopic observation. Finally, only five of the mutants had no scales coating the skin, and the PCR detection results showed that these mutants were all with bi-allelic insertion of 11 bp nucleotides. We further analyzed scale development using Alizarin red staining method. As shown in *Fig. 3*, scales coated the whole body of the wild type *(Fig. 3-A,B)*, while the F1 mutant *(Fig. 3-C,D)* contained just few scales on the body. Meanwhile, the *eda* expression pattern were assayed by Western blotting in both the mutant and wild zebrafish *(Fig. 3-E)*, with β-actin as the internal reference *(Fig. 3-F)*. We could see obvious

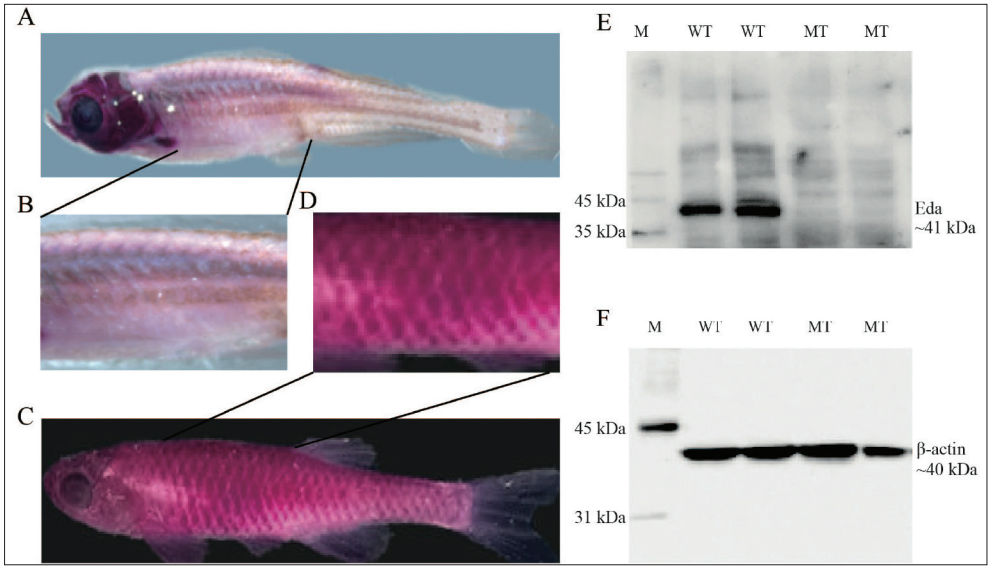

Fig 3. The Alizarin red staining and Eda expression assay of wild-type (WT) and mutation-type (MT) zebrafish. **A, B:** Individual containing an insertion 11 bp allelic mutation contains abnormal scaling when compared to the WT individual **(C, D)**. **E, F:** Western blotting of lysates from WT and the mutant carrying bi-allelic frameshift mutation with anti-Eda antibody and β-actin, respectively. M is the Unstained Protein Marker (NO. C600525 and NO. C600526, Sangon Biotech)

results as illustrated in *Fig. 3-E* that there is no targeted product in the mutants (MT) compared with that of the wild type (WT) zebrafish.

DISCUSSION

To date, a number of genes have been edited by the CRISPR-Cas9 system in zebrafish, and the mutation frequencies have been recorded up to 75% [12] and 86% [13]. By comparison, the mutation frequency of *eda* exon 4 was only 16% which was relatively low in the present work. And no mutations were detected in the embryos injected with sgRNA-2. It has proved that mutation frequencies depend on target loci in the zebrafish genome [11], which may be caused by the specific nucleotide of the targeting sequence in *eda*. Gene mutation include base substitution, deletion and insertion mutations, deletion and insertion mutations are the major mutations by the the CRISPR-Cas9 system introduced in zebrafish, especially deletion mutation [12-15]. In the paper, we obtained eight mutants, seven of them were deletion mutations, one of them is insertion mutation, however base substitutions were also occured with deletion and insertion mutations (shown in F0 6#, 7# and 8# of *Fig. 2*). In addition, a large relatively fragment of 87 bp deletion (F0 5#) was detected in this study, which was less common in other similar studies.

The above mutations, whichever are all likely to change the structure of protein. Five *eda* mutation types were detected in the embryos injected with sgRNA-1, the 6 bp deletion and the 11 bp insertion mutations resulted in the reading frame shift mutation of EDA protein. The frameshift mutations in the target gene are expected to affect the function of the EDA protein. However, the scale phenotype of all F0 mutation types were normal. Makino et al.[16] research suggested that all of the bi-allelic out-of-frame mutations expressed the target gene products are due to illegitimate translation. This means that the individuals with homozygous mutations of genes will produce a pronounced phenotype. In consequence, F1 generations are necessary to achieve bi-allelic mutants.

It is well known that homozygous mutant in F0 generations is hard to achieve; however, phenotyping could be done in F1 generation by inbreeding two founder fish [17], significantly reducing animal husbandry and time. In the study, five mutants were obtained without scales coating the skin, the results sugguested that bi-allelic 11-bp insertions in *eda* by PCR and sequecing detection. Western blotting were executed to further verify the function of EDA protein, the results showed that there was no expression product of EDA protein in scale-less fish compared with wild-type fish (shown in *Fig. 3*). We speculated that a frameshift mutation was caused by bi-allelic 11-bp insertions, and then leads to function inactivation of the CL and TNF domains. Similar experimental verifications have been performed in mouse and stickleback models [16], all of which suggested that changing the EDA signal pathways resulted in altered plate development in sticklebacks. Colosimo et al.[18] injected single-celled embryos from low-plated parents with full-length mouse EDA-A1 cDNA. The numbers of armor plates from the transgenic fish were then compared to the wild type fish, confirming that Eda signaling triggers lateral plate formation. Our results confirmed further that EDA was a crucial gene in scale development. Mutation in the *eda* leading to an out-frame mutation at the exon 4 locus affected scale development in zebrafish. The mutants will provide a good model for unveiling the role of EDA in fish scale development and elucidating the CL domain function. Before that, we need to consider the off-target effects of the CRISPR/Cas9 system as well as ZFN [19].

Acknowledgements

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Competing Interests

The authors declare that they have no competing interests.

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Research Article

Detection of BVDV 1q in China: Genetic Characterization and Experimental Infection for the Investigation of It's Pathogenicity

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Abstract

Bovine viral diarrhea virus (BVDV) is a pathogen that affects ruminants worldwide and is one of the most economically important diseases of cattle. Although BVDV infections have been increasingly reported in China, the pathogenesis and genetic characteristics of these BVDV isolates have not been thoroughly investigated. Here, we report the identification and characterization of a novel BVDV isolate, designated LC, which was isolated from the feces of a cattle with diarrhea. The complete genome of isolate LC was 12.271 nucleotides and contained a 5'-UTR of 389 nucleotides, a 3'-UTR of 189 nucleotides, and a large ORF encoding a polyprotein consisting of 3898 amino acids. Genomic comparisons and phylogenetic analyses of the complete genomic sequence clearly showed that the isolate was a BVDV-1q subtype. Experimental infection of calves with isolate LC resulted in the development of clinical signs including elevated rectal temperatures, nasal discharge and decreased leucopenia. Viral antigen was detected in infected animal tissues using immunohistochemistry. This is the first report of the genomic sequence of a BVDV-1q virus isolated from cattle. The virus strain was moderately pathogenic in calves and could potentially be used as a BVDV challenge virus to evaluate the efficacy of BVDV vaccines.

Keywords: *Bovine viral diarrhea virus, BVDV, Genomics, Genotyping, Pathogenesis*

Çin'de BVDV 1q'nun Tespiti: Patojenitesinin Araştırılması Amacıyla Genetik Karakterizasyon ve Deneysel Enfeksiyon

Öz

Sığır viral diyare virüsü (BVDV) dünya çapında ruminantları etkileyen ve sığırların ekonomik olarak en önemli hastalıklarından biri olan bir patojendir. BVDV enfeksiyonları Çin'de giderek daha fazla rapor edilmesine rağmen, BVDV izolatlarının patogenezi ve genetik özellikleri tam olarak araştırılmamıştır. Bu araştırmada, ishalli sığır dışkısından izole edilen ve LC olarak adlandırılan yeni bir BVDV izolatının tanımlanması ve karakterizasyonu rapor edildi. İzolat LC'nin tam genomu, 5'-kodlanmayan bölgesinde 389 nükleotid ve 3'-kodlanmayan bölgesinde 189 nükleotid içeren 12.271 nükleotitten oluşmaktaydı ve 3898 amino asitten oluşan bir poliproteini kodlayan büyük bir ORF içeriyordu. Tüm genomik dizinin genomik karşılaştırmaları ve filogenetik analizleri, izolatın bir BVDV-1q alt tipi olduğunu açıkça göstermiştir. İzolat LC ile buzağıların deneysel enfeksiyonu, rektal sıcaklık artışı, burun akıntısı ve lökopeni gibi klinik bulguların gelişmesine neden oldu. Viral antijen, enfekte hayvan dokularında immünohistokimyasal olarak tespit edildi. Bu çalışma, sığırlardan izole edilmiş bir BVDV-1q virüsünün genomik dizisinin ilk bulgusudur. Virüs suşu buzağılarda orta derecede patojenite göstermiştir ve BVDV aşılarının etkinliğini değerlendirmek için potansiyel bir BVDV bağışıklık virüsü olarak kullanılabilir.

Anahtar sözcükler: *Sığır viral diyare virüsü, BVDV, Genomik, Genotipleme, Patogenez*

INTRODUCTION

Bovine viral diarrhea virus (BVDV), the etiological agent of bovine viral diarrhea/mucosal disease, is a prevalent virus of economic importance in the cattle industry. BVDV-associated diseases range from clinically mild to severe and can involve the respiratory, enteric, reproductive, immune, and endocrine systems. BVDV belongs to the *Pestivirus*

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genus of the *Flaviviridae* family, the same genus as classical swine fever virus (CSFV) and border disease virus (BDV) of sheep [1,2].

The BVDV genome comprises single-stranded RNA of about 12.3~12.5 kb in length. The whole genome can be divided into the 5′-untranslated region (5′-UTR), a large open reading frame (ORF), and the 3′-untranslated region (3′-UTR). According to sequence comparisons of the BVDV 5′-UTR, BVDV was divided into two genotypes: bovine viral diarrhea virus type I (BVDV-1) and bovine viral diarrhea virus type II (BVDV-2) [3]. At present, according to comparisons of the 5′-UTR, N^{pro}, and E2 sequences in the BVDV genome, BVDV-1 can be divided into 22 subtypes (1a-1v), and BVDV-2 can be divided into four subtypes [4-7]. Because of the high mutation rate of RNA viruses and increasingly frequent international exchange and trade, new subtypes of BVDV are still emerging. Recently, an atypical bovine virulent BVDV-3 genotype was detected in bovine serum [8], which can be divided into two genetic subtypes *(Brazilian* source and *Thai* source), but the viral typing of BVDV-3 has not been confirmed according to the international classification of viruses. A comparative analysis of the 5′-UTR sequences of BVDV isolated from 2005 to 2013 in China was conducted. Most of the endemic strains in China were reported to be BVDV-1, and the main subtypes were BVDV-1b, BVDV-1m, and BVDV-1q [9-11].

According to the results of a BVDV epidemiological survey in China, more than 46.7% of cattle farms tested positive for BVDV antigen, and the persistent infection rate of BVDV in cattle herds was 2.2% [12-14]. In 2008-2010, in China's Xinjiang region, an epidemiological investigation of BVDV in district dairy farms revealed an average infection rate of 43.39%, with genotype BVDV1b being predominantly detected [15]. In another investigation of epidemic strains in Xinjiang in 2015, 2 of the 21 strains analyzed belonged to the BVDV-1b subtype, 15 strains had high homology with strain SD0803 of porcine BVDV isolated from Shandong, belonging to BVDV-1q, and the other four strains belonged to BVDV-2 [6]. The BVDV-1q subtype strain may have become an epidemic strain of BVDV in Xinjiang. The pathogenicity of BVDV-2 strains has been widely reported [16-18], but few studies have reported the pathogenicity of BVDV-1 strains.

In this study, one virus was isolated from fecal samples of cattle using MDBK cell cultures, and identified as a BVDV isolate by reverse transcriptase-polymerase chain reaction (RT-PCR) method and immunofluorescence assay. To investigate the genetic subgroup of the strain, the 5′-UTR and complete genome of the virus was sequenced and compared with other reference BVDV strains by phylogenetic analysis. The pathogenesis of the virus was evaluated by intranasally inoculating to susceptible calves to assess the potential endemic risk to the cattle herd in China. To understand the origin and evolution of BVDV strain and determine the molecular characters of the BVDV strains predominantly spread in China. The results confirmed the existence of the BVDV type 1q in China, and it may be helpful in preventing the BVD in China and a challenge virus strain for efficacy evaluations of vaccines.

MATERIAL and METHODS

Herd(s) History

A disease characterized by severe diarrhea occurred on a cattle farm with 420 cattle in Xinjiang province. The sick cattle were observed to manifest pyrexia,anorexia in early days, and later oral mucous ulcer and severe diarrhoea containing mucous and haemorrhage excretions found.

Approximately 15% (63/420) of cattle showed clinical signs. Treatment of the sick cattle with Penicillin or gentamycin yielded no effects on relieving the clinical signs. Blood samples obtained from cattle with diarrhea in Xinjiang Province, China in 2016 all tested with the IDEXX BVDV Ag/Serum Plus Test (Idexx Labs Inc, USA). Fecal samples were collected directly from the rectums of cattle to prevent contamination from the environment or between samples.

Virus Isolation

Fecal samples were diluted 1:9 (w/v) in sterile phosphate-buffered saline (PBS) with 1% antibiotics, and centrifuged at 10.000 × g for 10 min followed by filtration through a 0.22-μm filter. The filtrates were stored at -80°C until use for genomic analysis and virus isolation.

Virus isolation was conducted as described previously [19]. Briefly, MDBK cells were infected with the filtrates in a 12-well culture plate. The cell cultures were frozen and thawed three times and passaged two to three times at 5-day intervals. Every passage of MDBK culture was observed for 5 days, with the presence or absence of cytopathic effects being recorded.

Immunofluorescence Assay

To detect BVDV in the infected MDBK cultures, an immunofluorescent assay was conducted as follows [20]. Briefly, cell lysate was added to each of four wells of a 24-well MDBK tissue culture plate. Positive virus (NADL strain) and DMEM media (negative control) were also added to the four wells. After 24 h incubation at 37°C in a 5% CO_2 atmosphere, the plates were fixed in 4% cold polyoxymethylene and then washed with PBS. The fixed plates were incubated with fluorescein isothiocyanate (FITC)-conjugated polyclonal anti-BVDV (VMRD, Washington DC, USA) antibody, followed by a 1 h incubation in a 37°C humid box. The fluorescence signal was observed using a fluorescence inverted microscope (Zeiss Axioskop-40, Carl Zeiss, Jena, Germany).

RT-PCR Detection and Complete Genomic Sequence Analysis

Total RNA was extracted from the filtrate of ten samples and cell culture fluids of the virus isolates using a Mini BEST Viral

RNA/DNA Extraction Kit according to the manufacturer's instructions (TaKaRa Bio Inc, Beijing, China). The RNA was resuspended in 30 µL of DEPC-treated water. The extracted RNA was reverse-transcribed using the M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer. Eleven primer sets were designed to amplify overlapping regions of the complete BVDV genome *(Table 1)*. The amplified fragments were harvested and cloned into pMD19-T vector (TaKaRa Bio Inc, China). The three recombinant clones were submitted for DNA sequencing (Sangon Biotech (Shanghai) Co. Ltd, Shanghai, China). The retrieved sequences were edited and trimmed with the Edit Seq program in the DNAStar software (6.0). Clustal W was used to align the nucleotide sequences. Phylogenetic analysis was performed by the distance-based ClustalW method using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software.

Experimental Infection of Animals

Six to nine-month-old, healthy calves were obtained from a calf farm in Xinjiang, China. All animals were confirmed to be free of BVDV, infectious bovine rhinotracheitis virus, bovine parainfluenza virus, and *Mycoplasma bovis* infection using enzyme linked immunosorbent assay (ELISA) kits (Idexx Labs Inc., Maine, USA) and by reverse transcription PCR (RT-PCR) or PCR for nucleic acid detection [21,22]. As we expected, all animals were BVDV mRNA-free. All animal experiments were approved by The Shihezi State University Institutional Animal Care and Use Committee (NO.A2018-173-01).

Table 1. *Primers used for amplification of the complete genome sequence*

Primer Name	Sequence	Length
BVDV-A1F	GATCAATCTCTCGTATACAC	375bp
BVDV-A1R	GCCATGTACAGCAGAGAT3'	
BVDV-AF	ATGCCCTTAGTAGGACTAGC	1351bp
BVDV-AR	TCAATATTGTACCAGTTGCACCAACCATG	
BVDV-BF	GAAGGGATACAACGGGCAATGTT	1613bp
BVDV-BR	ACCATCTGGAAGGCCGGTCCATTCAGCA	
BVDV-CF	ATGGATGACAACTTTGAATTTGG	1167bp
BVDV-CR	ACCAGTGGCACTATAGTCGGGTC	
BVDV-DF	GTGATGATGGGCAACTTGCTAACACATGAT	1286bp
BVDV-DR	CATTTTGGGCAGGTGCCACCTTTCCACTT	
BVDV-EF	AACCTAATAATAAAACATAAGGT	1431bp
BVDV-ER	AGTTCTGTGGTTTTTCCTGCCCCTGTTGC	
BVDV-FF	AGGGTGGTTGGCAGAGTTAAGGTAGGGAAG	1288bp
BVDV-FR	TATGCAAGTTGGATTGGCTCTGGGTG	
BVDV-GF	TATTATAGGAGCCAGGAAACAGCAAC	1499bp
BVDV-GR	TGATCCAAGAAGTTCTTTACAAACACCTTCAT	
BVDV-HF	ATGGAAATCCTGTCACAAAACCCAGT	1451bp
BVDV-HR	TTTGCTCTATTTGACATGGAACTC	
BVDV-IF	ACATCAGAGGTTGGGATCACAATAATTGG	1466bp
BVDV-IR	TCATTTTTTGGTATTGCTGTTTCATAATA	
BVDV-JF	TAAAACACACCTATGGTGAGGTGACGTGGGA	1746bp
BVDV-JR	CCTCATACAGCTAAAGTGCTGTGTGCATT	

Animal experiments are conducted in the veterinary biosafety level 3 laboratory (BL3) of Xinjiang Tiankang Biological Technology Co. Ltd. Infected animals are raised in the biosafety level III equipment (negative pressure isolator), and all operations are conducted in the biosafety level III cabinet. The six calves were divided randomly into treatment and control groups, with three animals in each group. Each calf in the treatment group was inoculated intranasally (IN) with ~$6\times10^{7.0}$ tissue culture infective dose ($TCID_{50}$) of the isolate BVDV LC virus. The animals in the control group were inoculated IN with DMEM. All animals were monitored daily for clinical signs as described previously [23]. Clinical assessments were made at the same time each morning by investigators who were blinded to the treatment groups. Clinical signs included depression, nasal discharge, diarrhea, coughing, and high rectal temperature. EDTA-blood samples from calves were collected at days -2 to 0 prior to inoculation and 2, 4, 6, 8, 10, 12, 14 and 16 days' post-inoculation (dpi) and were used to count white blood cells. Additionally, at -2, 0, 2, 4, 6, 8, 10, 12, 14 and 16 dpi, heparin blood was sampled for buffy-coat preparations to test for viremia in the infected calves. Deep nasal swab specimens were obtained from 1 day prior to challenge through to 14 dpi. The procedure to isolate BVDV from samples was conducted as described previously [19,24]. Two calves from each group were necropsied at 16 dpi, and tissue samples of liver, spleen, lung, heart, kidney, intestine and mesenteric lymph node were collected and fixed in 10% neutral buffered formalin and processed for histopathological examination following hematoxylin and eosin (H&E) staining and immunohistochemistry. After the animal experiment is finished, animal carcasses and all animal indoor wastes should be autoclaved before incineration or other final treatment.

Data Analysis

The two groups (treatment and control) were analyzed and compared with respect to the primary clinical signs including rectal temperature, nasal and ocular discharge, diarrhea, leukopenia, and virus shedding, using GraphPad Prism (version 4.0) software. The level of statistical significance was set at $P<0.05$.

RESULTS

BVDV is the Causative Agents for the Outbreak

No obvious effect was observed the majority of sick cattle after they were treated with Penicillin or gentamycin, suggesting the outbreak is likely associated with viral agents. All samples were tested by the IDEXX BVDV Ag/Serum Plus Test (Idexx Labs Inc, USA), 420 antibody postive samples were detected, the positive rate of BVDV antibody

was 100% (420/420). Collected blood samples were tested by RT-PCR, 98 positive samples were detected, approximately 23% (98/420) of BVDV antigen positive rates. The sequencing analysis of 5′-UTR fragment amplified by RT-PCR showed that the sick cattle were infected with BVDV virus.

BVDV-LC is a Noncytopathic Biotype BVDV Isolate

Fecal samples were cultured and passaged in MDBK cells, and no cytopathic effects were observed in MDBK cells after 15 passages. However, specific fluorescence signals were detected in the cells inoculated with 15 passage supernatants using FITC-labeled BVDV antibody, whereas no fluorescence signals were detected in the mock infected cells *(Fig. 1)*. To confirm these results, PCR was performed and fragments were amplified from the infection group, and sequencing revealed that the sequences were BVDV-specific (data not shown). No fragments were amplified from the control group. One viral isolate was obtained from the fecal samples and this strain was designated as BVDV-LC.

Sequence and Phylogenetic Analysis of the Complete Genome

To investigate the evolutionary relationship between BVDV isolates, phylogenetic analysis was performed using MEGA software version 6.0. A phylogenetic tree constructed based on full-length genome sequences revealed that isolate BVDV-LC clustered with previous BVDV-1 isolates *(Fig. 2-A)*. The genome sequence of BVDV-LC shared nucleotide sequence identities of 63.0% to 95.1% with other *Pestivirus* strains, 70.8% identity with BVDV-2 strains, 69.1% identity with BVDV-3, 67.9% with CSFV strains, and 63% identity with the pronghorn strain (AY781152 antelope). The complete genome sequence of isolate LC was compared with those of nine BVDV-1 strains, three BVDV-2 strains, one strain of Pronghorn antelope pestivirus, two strains of BDV, two strains of CFSV, and one strain of BVDV-3 *(Table 2)*. The sequences of BVDV-1 viruses showed a higher degree of divergence from that of isolate LC than from those of the BVDV-2 strains. In the coding sequences, the highest degree of shared identity was observed between the LC and SD0803 strains. The full-length genome sequence of SD0803 was 95.1% identical to that of the LC isolate.

To confirm the subtype assignment based on the full-length genome sequence and to compare our isolates to other reference strains, phylogenetic trees based on the 5′-UTR and N^{pro} genes were constructed *(Fig. 2-B,C)*. For this analysis, sequences from members of 21 genetic subgroups of BVDV-1 from different regions of the world were used. The two phylogenetic trees showed that isolate BVDV-LC clustered in the same phylogenetic branch as in the phylogenetic tree based on the full-length genome *(Fig. 2-B,C)*. The 5′-UTRs of the Shihezi148-1, SD0803, Changji, and Kuerle virus strains shared 92.5% and 98.5% sequence identity, respectively, to the isolate in the present study. The N^{pro} sequence of the SD0803 isolates shared 92.7% sequence identity with the BVDV-LC isolate. Furthermore, the BVDV-LC isolate clustered with strain SD0803, which belongs to the BVDV-1q subgenotype [25].

The complete genome of the BVDV-LC strain of BVDV-1(GenBank accession No. MK102095) was 12.271 kb in length and contained a single ORF that was 11.694 kb in length, encoding a 3898-amino-acid polypeptide. The 5′ and 3′-UTRs of the BVDV-LC virus were 388 and 186 bp in length,

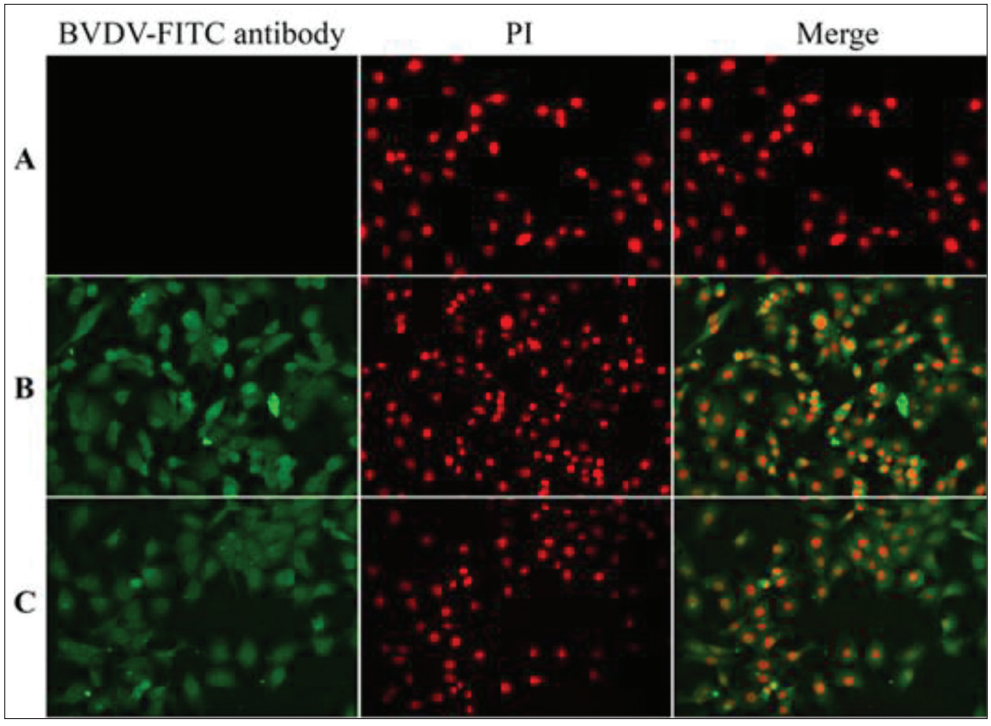

Fig 1. Immunofluorescent assay to detect BVDV-LC in cultured MDBK cells. **A:** Control cells; **B:** NADL-positive control; C: BVDV-LC

Table 2. *Nucleotide sequence identity (%) of the BVDV-LC strain to the coding sequences of other whole genome sequences of Pestivirus isolates used in the trees*

Strain	Pestivirus	N^{pro}	C	E^{rns}	E1	E2	P7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
Singer-Arg	BVDV-1	81.3	76.1	80.9	77.6	71.7	74.8	78.0	83.0	81.8	81.8	76.9	79.6
NADL		83.3	77.5	80.2	77.3	72.5	73.8	74.8	83.6	80.7	82.2	76.5	79.9
SD1		82.3	78.1	80.3	77.9	72.6	76.2	79.6	83.3	83.9	81.8	76.5	79.5
KS86-1		82.3	79.1	80.2	77.6	74.2	78.1	77.9	83.0	82.3	83.0	76.1	80.7
CP7		80.2	78.8	79.6	76.9	73.4	74.3	76.3	82.6	83.3	79.7	76.5	80.8
VEDEVAC		79.6	80.1	79.1	75.6	72.9	71.4	75.9	82.3	82.3	79.9	76.6	79.9
KE9		79.2	77.5	81.4	75.9	73.4	78.6	76.8	81.6	84.4	81.4	76.9	79.7
Zm95		81.9	77.5	84.0	81.5	77.5	81.4	80.8	85.6	84.9	85.8	82.0	82.6
SD0803		92.7	95.1	94.7	94.4	92.0	94.3	94.8	96.5	95.3	95.9	96.4	96.0
SH-28	BVDV-2	70.2	66.3	71.1	71.1	64.4	59.0	57.6	78.2	75	75.8	66.4	70.2
New York93		71.8	66.7	70.8	69.7	57.9	60	62.0	78.5	74.0	74.9	65.1	70.9
890		72.4	67.6	71.4	69.7	65.5	61.9	57.1	77.9	74.0	75.0	66.6	70.3
Antelop	Pestivirus	59.3	65.0	45.2	57.9	56.2	44.3	37.7	71.0	55.2	67.7	57.7	62.5
X818	BDV	66.9	59.5	70.5	69.1	63.2	56.2	59.4	77.7	76.6	65.8	59.3	70.1
BD31		64.7	63.4	71.5	67.0	61.1	55.2	58.0	76.9	74.5	66.8	62.6	69.6
cf114	CFSV	66.5	63.4	67.5	68.4	61.6	56.2	60.2	76.6	75.5	69.0	61.6	69.4
Shimen-HVRI		65.9	63.1	67.5	68.2	59.9	56.7	60.4	76.6	75.5	69.0	61.7	69.4
Th-04-KhonKaen	BVDV-3	67.3	66.7	73.7	69.7	61.9	52.4	54.8	77.1	77.1	73.6	62.0	70.8

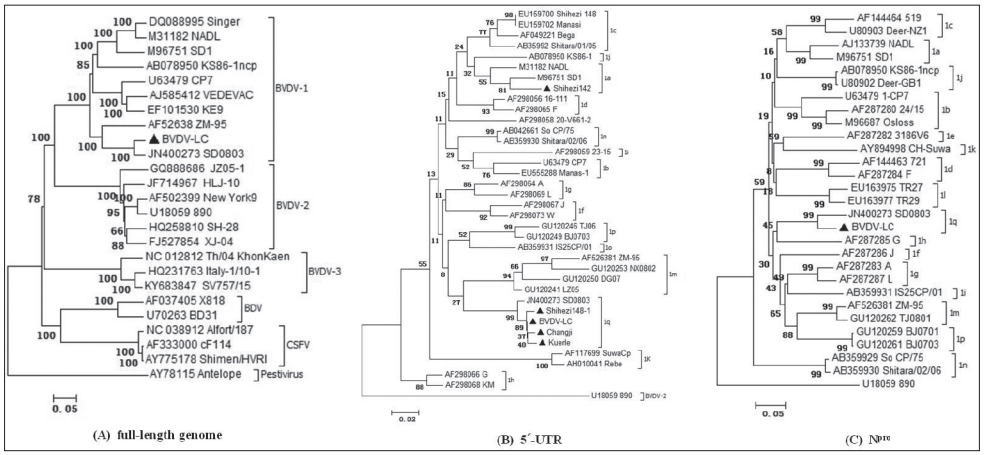

Fig 2. Phylogenetic trees based on the nucleotide sequences of (A) the full length genome, (B) the 5′-UTR and (C) the N^{pro} gene. Phylogenetic tree analysis of each gene was prepared using the Neighbor-Joining method and bootstrap analysis (1.000 replicates) using the software MEGA v. 6.0

respectively. Based on the putative post-transcriptional processing sites of other BVDVs, the SD0803 genes and UTRs were mapped to the following nucleotide positions in the genome: 5′-UTR(1-388), N^{pro}(389-892), C(893-1197), E^{rns}(1198-1879), E1(1880-2464), E2(2465-3586), p7(3587-3795), NS2-3(3796-7204), NS4A(7205-7396), NS4B(7397-8437), NS5A(8438-9925), NS5B(9926-12082), and 3′-UTR (12083-12271).

Pathogenesis of Infection in Cattle Experimentally Infected with BVDV 1q

All three calves inoculated with BVDV-LC developed moderate clinical signs associated with BVDV infection, including depression, fever, leukopenia, and viremia. No clinical signs were observed in the control calves. The rectal temperatures of the calves were measured daily from day -2 (before inoculation) to day 16 (after inoculation). All calves in the treatment group had elevated rectal temperatures after inoculation, and the highest temperature was over 40 degrees on day 8 to day 12. In the control group, there was no increase in the rectal temperature of the calves *(Fig. 3-A)*. In the treatment group, the number of white blood cells began to decline on the second day after inoculation, and decreased the least on the sixth day after injection, to almost 40%. There was no change in the number of white blood cells in the control group *(Fig. 3-B)*.

In the treatment group, the results of viral detection were positive from 4 to 12 dpi in nasal and blood samples. Viral shedding was detected by virus isolation in MDBK cells from nasal swabs and blood samples. Two of the infected calves showed viral shedding as early as 4 dpi and the longest shedding period reached 12 days after inoculation. Results showed that all infected calves had viremia and BVDV was isolated from blood from different calves on

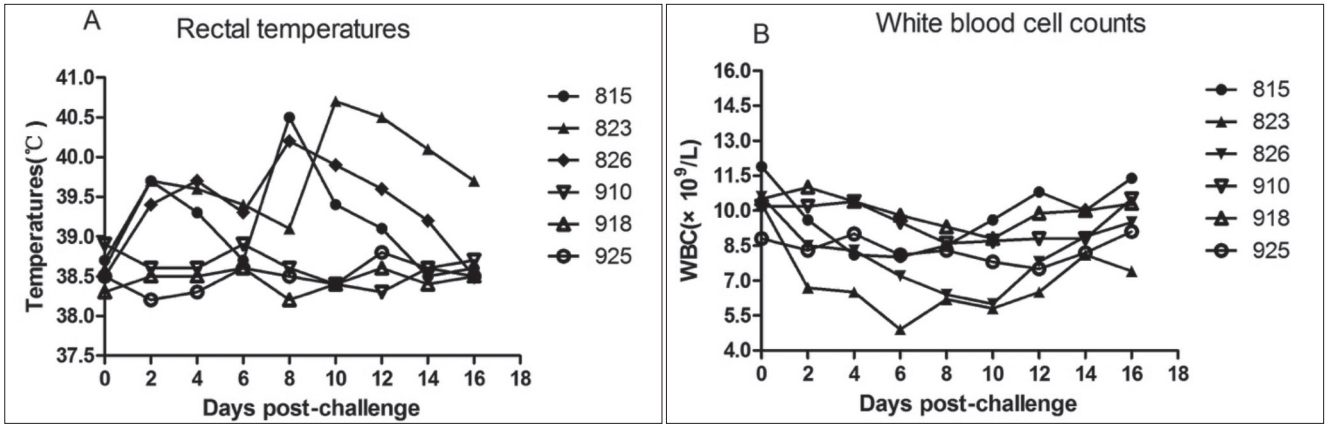

Fig 3. The clinical observation following inoculated with BVDV-LC virus. A: Elevated rectal temperatures were detected, B: Decreased white blood cell counts were also detected

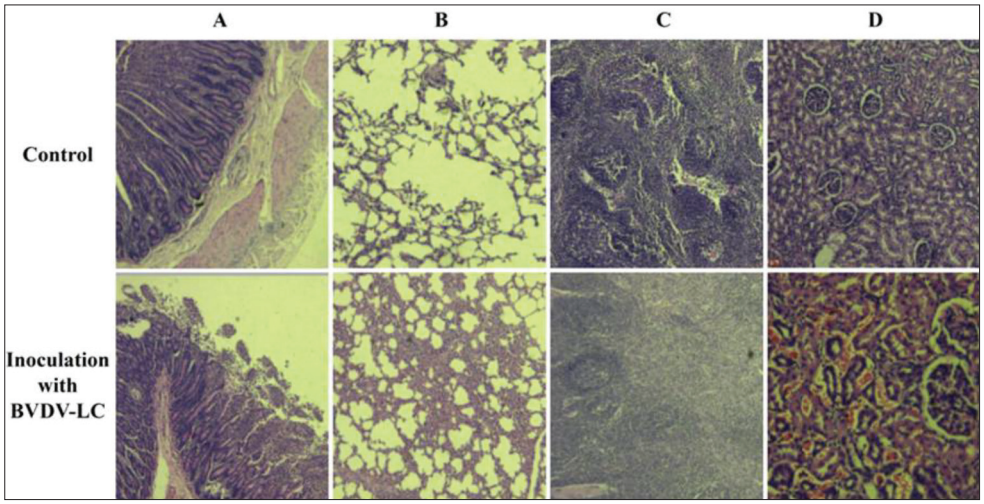

Fig 4. Histopathology of calves inoculated with BVDV-LC isolate. **A:** Villus rupture in the small intestine, epithelial cell shedding, and basal layer edema; The control was normal, **B:** Pulmonary interstitial broadening and interstitial pneumonia; The control was normal, **C:** Reduced spleen nodules; The control was normal, **D:** Renal tubular interstitial hemorrhage and hyperemia of glomerular capillaries; The control was normal

days 4-12 days. Sera obtained before inoculation with BVDV-LC and on day 14 was subjected to neutralization testing. Results showed that all of the virus-infected calves developed virus-neutralizing antibodies by day 14. The results of viral detection were negative after inoculation in the control group.

All experiment calves were euthanized at 16 dpi, one mock-infected calf (918) and two randomly chosen infected calves (815 and 823) for histopathology analysis. Gross pathological findings included enlarged mesenteric lymph nodes, obvious renal hemorrhage, intestinal inflammation, and severe intestinal hemorrhage. Compared with the control group, all of the organs in the challenge group showed obvious pathological changes, including: villus rupture in the small intestine, epithelial cell shedding, and basal layer edema *(Fig. 4-A);* pulmonary interstitial broadening and interstitial pneumonia *(Fig. 4-B);* reduced spleen nodules *(Fig. 4-C);* renal tubular interstitial hemorrhage and hyperemia of glomerular capillaries *(Fig. 4-D);* and finally, no pathological changes in the heart. The central venous and interlobular veins of the bovine liver were filled with blood.

Immunohistochemical staining was performed on the tissues of the heart, liver, spleen, lung, kidney and small intestine of calves in the challenge group. Virus infection was detected in the liver and lung, which had low viral loads, and the small intestine and heart tissues, which showed higher viral loads *(Fig. 5)*. In the control group, there were no signs of virus infection in any of the tissues.

DISCUSSION

In this study, a new BVDV-LC strain was isolated from calves afflicted with bovine viral diarrhea/mucosal disease, which was similar to a BVDV isolated in Xinjiang Province, China in 2016. The pathogenesis of this isolate was tested by experimental infection. A virus of this genetic subtype has previously been detected in pigs in China [25]. BVDV1-q was isolated from cattle and pigs, suggesting that this virus has evolved to replicate well in different species. At present, there are 22 subtypes of BVDV1, and the diversity of genotypes is one of the main features of BVDV. Genetic evolutionary analysis is an effective tool for tracing the origin of newly emerged viruses for epidemiological and vaccine research [26-28].

A previous study indicated that the BVDV 1a subtype was

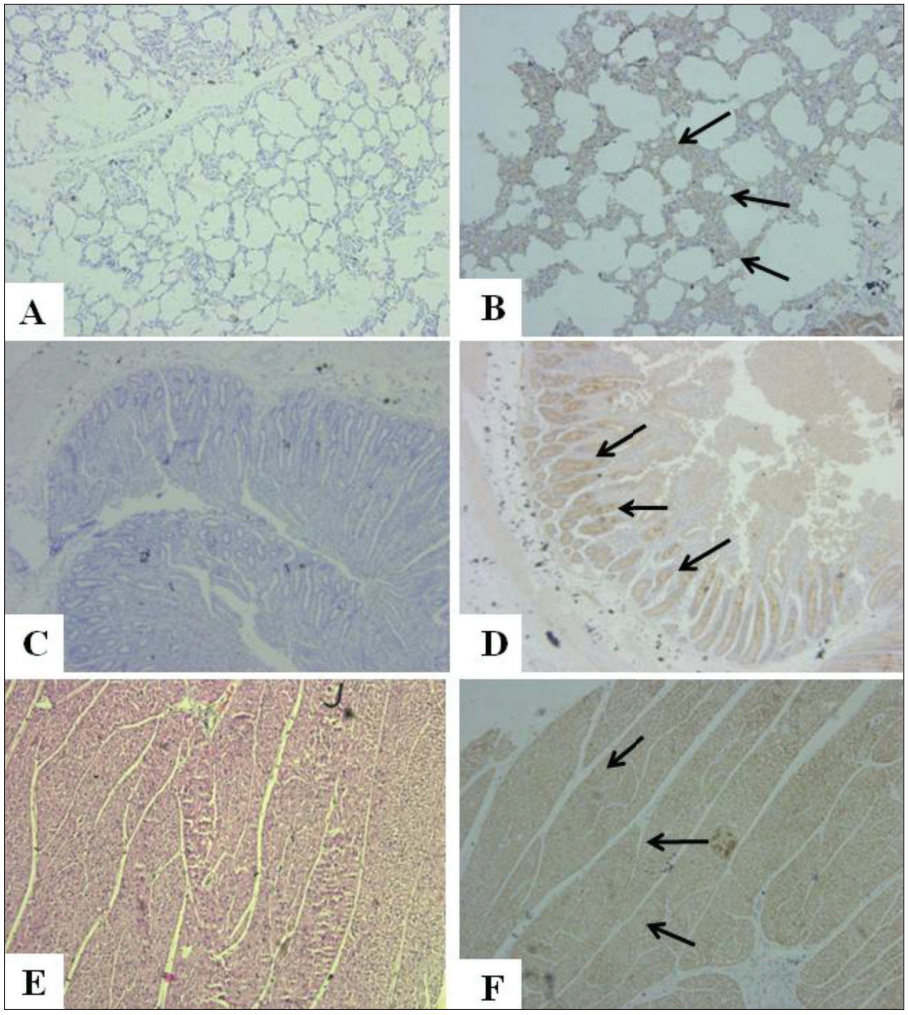

Fig 5. Immunohistochemical analysis of the tissues of calves inoculated with BVDV-LC isolate. **A:** Section of the lung of a mock-infected calf. There is no signal, **B:** Section of the lung of a calf experimentally infected with BVDV-LC. Hybridization signals are detected by anti-BVDV antibody *(arrows),* **C:** Section of intestinum tenue of a mock-infected calf. There is no signal, **D:** Section of the intestinum tenue of a calf experimentally infected with BVDV-LC. Hybridization signals are detected by anti-BVDV antibody *(arrows),* **E:** Section of heart of a mock-infected calf. There is no signal, **F:** Section of the heart of a calf experimentally infected with BVDV-LC. Hybridization signals are detected by anti-BVDV antibody *(arrows)*

predominant and widespread in countries neighboring China, such as Korea and Japan [29,30], whereas, in China and India, BVDV 1b was the predominant subgenotype [23,31]. Four isolates of BVDV, the Shihezi148-1, Changji, Kuerle, and SD0803 viruses, were recently isolated from a dairy in northwestern China. All four isolates were classified as belonging to a potentially novel subgenotype, and a partial 5′-UTR sequence of each isolate was obtained. To determine the genetic relationship between the BVDV-LC virus and these novel subgenotypes, a phylogenetic tree was constructed based on the 5′-UTR sequences, which showed a single branch containing four isolates clustered with the BVDV-LC virus *(Fig. 1B)*. The 5′-UTR of the SD0803 virus shared a high degree of sequence homology (98.5%) with the SD0803 viruses, which indicates that these viruses should thus be classified as strains of the BVDV-1q subgenotype. Similar to the phylogenetic tree based on the N^{pro} sequences, the BVDV-1q branch was most closely related to the BVDV-1m, BVDV-1o, BVDV-1p, and BVDV-1g clusters in the 5′-UTR-based phylogenetic tree.

In this study, the pathogenesis of BVDV-LC was studied and only mild clinical symptoms associated with BVDV infection were observed. These included depression, fever, leukopenia, and viremia. Calves infected with the virus showed no symptoms of diarrhea. Furthermore, the experimental animals showed no secondary infections with other pathogenic microorganisms such as pasteurellosis or *Escherichia coli*. Histopathological observations showed lymphoid depletion and shedding of the villi of the small intestine. Many studies have reported that animals infected with non BVDV show leukocyte depletion, failure to induce the production of type I interferon α/β, immune suppression, and often secondary infections [32-35].

In summary, a BVDV-LC strain was successfully isolated from cattle in northwest China. We determined the complete genome sequence of the cattle-derived LC strain of BVDV-1 and phylogenetic and sequence analyses based on the N^{pro} gene and the 5′-UTR showed that the BVDV-LC strain belongs to the BVDV-1q subgenotype. This is the first report of the genomic sequence of a BVDV-1q virus isolated from cattle. Calves inoculated with this isolate showed mild clinical signs, a high rectal temperature, and lymphopenia, which suggested that the strain remains virulent and can be used as a BVDV challenge virus to evaluate the efficacy of BVDV vaccines. This report forms a solid basis for further studies on BVDV in China.

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Research Article

Effects of Cleavers *(Galium aparine)* and Yarrow *(Achillea millefolium)* Extracts on Rumen Microbial Fermentation in *In-vitro* Semi-Continuous Culture System (RUSITEC)

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Abstract

Experimental data on the effects of cleavers *(Galium aparine)* and yarrow *(Achillea millefolium)* extracts on rumen microbial fermentation are scarce. The objective of this study was to determine the effects of cleavers and yarrow extracts on *in vitro* ruminal fermentation. Incubation trial was carried out using the long-term rumen simulation technique (RUSITEC). The experiment lasted 10 days. After an adaptation period of 5 days, the fermentation vessels divided into 3 groups; first three vessels received no additives (control), second three vessels received 500 mg/L cleavers extract daily, and third three vessels received 500 mg/L yarrow extract daily. Supplementations of cleavers and yarrow extracts had no significant effect on ruminal pH, total volatile fatty acids (VFA), acetate, propionate and methane production, NH_3-N concentration and, total protozoa. However, both extracts decreased dry matter digestibility (DMD) ($P<0.05$). Butyrate production, on the other hand, increased with cleavers extract ($P<0.05$). In conclusion, there were only small effects of cleavers and yarrow extracts on the investigated microbial fermentation characteristics. Nevertheless, it may be considered advantageous for feed conversion that plant extracts did not suppress ruminal fermentation in spite of decreasing DMD.

Keywords: *Achillea millefolium, Cleavers, Galium aparine, Plant extracts, Rumen, RUSITEC, Yarrow*

Yoğurt Otu *(Galium aparine)* ve Civan Perçemi *(Achillea millefolium)* Ekstraktlarının *In-vitro* Yarı-Sürekli Kültür Sisteminde (RUSITEC) Rumen Mikrobiyal Fermentasyonu Üzerine Etkileri

Öz

Yoğurt otu *(Galium aparine)* ve civan perçemi *(Achillea millefolium)* ekstraktlarının rumen mikrobiyal fermentasyonu üzerine etkileri ile ilgili sınırlı düzeyde deneysel veri bulunmaktadır. Bu çalışmanın amacı, yoğurt otu ve civan perçemi ekstraktlarının *in vitro* ruminal fermentasyon üzerine etkilerinin araştırılmasıdır. Inkübasyon denemesi uzun-süreli rumen simülasyon tekniği (RUSITEC) kullanılarak gerçekleştirilmiştir. Deneme 10 gün sürmüştür. Beş günlük bir adaptasyon periyodunu takiben fermenterler 3 gruba bölünmüştür; ilk üç fermentere ilave yapılmamış (kontrol), ikinci üç fermenterlik gruba günlük 500 mg/L yoğurt otu ekstraktı, üçüncü üç fermenterlik gruba ise günlük 500 mg/L civan perçemi ekstraktı eklenmiştir. Yoğurt otu ve civan perçemi ilaveleri, ruminal pH, toplam uçucu yağ asitleri (UYA), asetat, propiyonat ve metan üretimi, NH_3-N konsantrasyonu ve toplam protozoa üzerine önemli bir etki oluşturmamıştır. Ancak her iki ekstrakt da kuru madde sindirilebilirliğini (KMS) azaltmıştır ($P<0.05$). Diğer taraftan, bütirat üretimi, yoğurt otu ekstraktı ilavesi ile artış göstermiştir ($P<0.05$). Sonuç olarak, incelenen mikrobiyal fermentasyon özellikleri üzerine yoğurt otu ve civan perçemi ekstraktları sadece küçük etkiler oluşturmuşlardır. Bununla birlikte, bitki ekstraktlarının KMS'yi azaltmalarına rağmen ruminal fermentasyonu baskılamamış olmalarının, yem maddelerinin değerlendirilmesi açısından avantaj olarak kabul edilebileceği düşünülmektedir.

Anahtar sözcükler: *Achillea millefolium, Bitki ekstraktları, Civan perçemi, Galium aparine, Rumen, RUSITEC, Yoğurt otu*

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INTRODUCTION

Manipulation of rumen microbial fermentation for enhancing feed digestibility, mitigating methane emission and nitrogen excretion by ruminants to improve animal productivity and to lower product cost is one of the main aims of nutritional strategies. Stabilizing ruminal pH to reduce the incidence of metabolic disorders such as subacute ruminal acidosis has long been another target for the rumen nutritionists [1]. Antibiotic feed additives which selectively inhibit Gram-positive rumen bacteria and rumen protozoa have been successfully used in ruminant rations for these purposes [2]. However, the risk of residues in animal products as well as the concern about the appearance of resistant strains of bacteria led to the prohibition of antibiotic use in animal feeds in the European Union [3] and Turkey [4] since 2006. Accordingly, the focus of researchers has shifted to the study of natural alternatives such as plant extracts and their secondary metabolites to manipulate ruminal fermentation in order to improve ruminant productivity [5]. Many of the plant secondary compounds in the extracts are well defined as antimicrobial agents which act against bacteria, protozoa and fungi [6]. In a new study, we have observed that green leaf volatiles which are derived from unsaturated fatty acids by plants with green leaves had favorable effects on protein utilization in the rumen via suppressing rumen protozoa [7].

Cleavers *(Galium aparine)* and yarrow *(Achillea millefolium)* are important medicinal plants which have been used for centuries to treat various diseases [8,9]. Cleavers grows widespread in Anatolia [8] and extracts of this plant are widely used in the treatment of sepsis, skin infections, and infections of respiratory and genitourinary systems due to antimicrobial properties [10]. The main biologically active antimicrobial phenolic substances in cleaver extract were chlorogenic acid, caffeic acid, and rutin [11]. It has been reported that Gram-positive bacteria mainly *Staphylococcus aureus* and *Bacillus subtilis* are more sensitive to cleavers extract than Gram-negative species such as *Escherichia coli* and *Proteus vulgaris* [10]. Yarrow also has antimicrobial activity and thus it is one of the most commonly used herbs in Europe in traditional animal healthcare and livestock production. Gastroprotective, antiulcerogenic, and anti-inflammatory properties of yarrow extract have been reported [9]. Yarrow extract was rich in antimicrobial phenolic compounds such as chlorogenic acid, vicenin-2, luteolin-7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside, luteolin, and apigenin [12]. *Staphylococcus aureus* and *Streptococcus pneumoniae* among Gram-positive wound pathogens have been reported to be more sensitive to yarrow extract than *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa* as Gram-negative pathogen species [13]. Yarrow essential oil has been also reported to be an effective antimicrobial agent against *Listeria* species [14] and a protozoan parasite *Trypanosoma cruzi* [15]. Antibiotic feed additives such as monensin produce desirable effects on rumen fermentation by showing selective antimicrobial activity on Gram-positive bacteria and protozoa in the rumen [2]. Therefore, the above-reported effects of these plant extracts on Gram-positive bacteria and protozoa suggest that they may be alternatives to antibiotics as feed additives in the modification of ruminal fermentation if they act in the same way in the rumen. However, there is limited information about the effects of yarrow extract on rumen fermentation. The ability of cleavers extract to influence microbial fermentation processes in the rumen have not been evaluated. Therefore, the aim of this study was to investigate the effects of cleavers and yarrow extracts on rumen microbial fermentation in an *in vitro* semi-continuous culture system (RUSITEC).

MATERIAL and METHODS

Plant Extracts

The extracts were provided by Kale Naturel Herbal Products Company, Ltd., Balıkesir, Turkey. As specified by the manufacturer, plant samples were air dried, ground in a mill into 0 to 200 µm large particles and screened. Powdered plant materials were extracted with 80% ethanol (1/10, w/v) at 30°C for 4-5 h and filtered to give homogenous liquid. The extracts was reduced to 1/5 of its volume using a rotary vacuum evaporator at 35°C for 8 h and dried in a laboratory scale spray-dryer. Afterwards, dry extracts liquefied in the mixer at an adequate ratio.

Analyses of Phenolic Compounds of Plant Extracts

Phenolic compounds *(Table 1)* of cleavers and yarrow extracts were quantified using a high-performance liquid chromatography (HPLC) (Shimadzu) device equipped with a photodiode array detector. An Agilent Eclipse XDB-C18 (250 × 4.60 mm) 5 µm column at 30°C and 0.8 mL/min flow speed was used.

Artificial Rumen System

The present study was carried out using the rumen simulation technique (RUSITEC) as described by Czerkawski and Breckenridge [16] applying slight modification according to Oeztuerk et al.[17]. The complete unit consisted of nine 1 L volume anaerobic fermenters. The inoculum was obtained from two freshly slaughtered beef cattle (400 kg mean body weight) at a commercial slaughter facility and transferred in warm (39°C) insulated flasks to the *in vitro* system within 30 min. According to information obtained from the owner, the animals had been fed a diet (8.0 kg DM/d) consisting of 20% barley straw and 80% commercial mineral- and vitamin supplemented concentrate for growing cattle. The same diet was also used for *in vitro* incubation trials *(Table 2)*. The commercial concentrate consisted of corn, wheat bran, corn gluten feed, molasses, sunflower seed meal, barley, distilled corn grain residues, soya bean meal,

Table 1. *Phenolic compounds of cleavers (G. aparine) and yarrow (A. millefolium) extracts*

Phenolic Compounds (ppm)	Cleavers Extract	Yarrow Extract
Gallic acid	ND	4.7
Protocatechuic acid	1.7	70.0
P-hydroxy benzoic acid	23.6	13.8
Chlorogenic acid	10.5	72.2
Caffeic acid	13.3	65.9
Syringic acid	2.4	13.0
Vanilin	1.6	18.6
P-coumaric acid	1.9	9.0
Ferulic acid	1.1	27.6
Benzoic acid	30.1	29.3
O-coumaric acid	0.6	6.7
Eriodictiol	ND	36.2
Apigenin	23.6	43.8
ND: not determined		

Table 2. *Chemical composition of experimental diet*

Nutrients, %	Barley Straw	Concentrate
Dry matter	94.19	91.12
Ash	5.82	6.50
Crude fiber	40.45	5.56
Crude protein	2.95	12.42
Ether extract	2.00	2.75
Organic matter	88.37	84.62
None fiber carbohydrates	16.43	61.44
Neutral detergent fiber	72.80	16.89
Acid detergent fiber	49.98	7.41
Metabolizable energy (MJ/kg)	6.28	11.11

vinasse, vegetable oil, calcium carbonate, sodium chloride, and a vitamin-mineral premix. Ruminal fluid was filtered through four layers of cheesecloth to partition into liquid and solid (digesta) fractions. Each fermentation vessel was filled with 750 mL of filtered ruminal fluid. Squeezed solid digesta (80 g) was weighed into a nylon bag (80 × 120 mm; 150 µm pore size), and placed in the inner perforated containers together with 10 g of an experimental diet (5 g barley straw and 5 g concentrate on feed basis). After 24 h, the solid digesta bag was withdrawn and a bag with feed was supplied. Thereafter, one feed bag was replaced daily, so that each feed bag remained in the fermentation vessel for 48 h. Fermentation vessels received a continuous infusion of a buffer (pH 7.4) at a rate of 750 mL/d [17]. The chemical composition of the buffer solution is presented in *Table 3*.

Experimental Design

The experiment was conducted as a completely randomized design (CRD) with three treatments and three replicates per treatment. The incubation trial consisted of a 5-day adaptation period (to achieve steady-state conditions) followed by a 5-day collection period. At the start of the collection period, the commercial extracts of cleavers *(G. aparine)* or yarrow *(A. millefolium)* were added to the respective fermentation vessels. Although there is no literature that evaluates effects of cleavers extract on rumen fermentation, yarrow extract was supplied at the rate of 500 mg per day in 1 L dual outflow fermenters by Broudiscou et al.[18]. Therefore, 500 mg/L dose of both extracts were used in the present study. During the collection period, the 9 vessels were divided into 3 groups: three of them received daily 500 mg/L of cleavers extract, three vessels received daily 500 mg/L of yarrow extract and three vessels received no additives (control).

Table 3. *Chemical composition of the buffer solution*

Chemicals	mmol/L
NaCl	28.00
KCl	7.69
1N HCl	0.50
$CaCl_2 \cdot 2H_2O$	0.22
$MgCl_2 \cdot 6H_2O$	0.63
$Na_2HPO_4 \cdot 12H_2O$	10.00
$NaH_2PO_4 \cdot H_2O$	10.00
$NaHCO_3$	97.90

Chemical Composition Analysis of Feed

The dry matter, crude protein, crude fat, crude cellulose, and ash contents of experimental diets *(Table 2)* were analyzed according to the procedure of the Association of Official Analytical Chemists [19]. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured [20] using an ANKOM^{200} Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA). NDF, using sodium sulfite, heat stable amylase, and ADF were expressed including residual ash. Non-fiber carbohydrates (NFC) were calculated as follows: NFC% = (100% - CP% - NDF% - EE% - Ash%) [21]. Organic matter (OM) content was determined based on the value of dry matter content minus ash content and metabolizable energy according to TSE [22] methods.

Sample Collection and Analytical Procedures

The pH values were measured daily in each fermentation vessel at the time of feeding using an epoxy body pH electrode (WD-35801-00, Oakton) connected to a pH-meter (Ion 6, Acorn series, Oakton).

Liquid effluent was collected daily and samples were taken and frozen at -20°C for later analyses for volatile fatty acids (VFA) and NH_3-N. VFA were quantified by the method of Ozturk et al.[23] using HPLC (Shimadzu LC-20AT) with a Rezex ROA-organic acid column (7.8 × 300 mm) at 60°C,

isocratic elution with 0.005 M H_2SO_4, and UV detection at 210 nm. NH_3-N concentration was determined using a colorimetric technique as described by Bhandari et al.[24].

Methane production was calculated using the equations proposed by Abdl-Rahman [25] based on the stoichiometry of Wolin [26] as following:

Fermentative CO_2 = A/2 + P/4 + 1.5 B

Fermentative CH_4 = (A + 2 B) – CO_2

A = mole of acetate

P = mole of propionate

B = mole of butyrate

Dry matter was determined by drying bags at 65°C for 48 h. The digestibility of dry matter at 48 h was calculated as original dry matter sample weight minus dry matter residue weight divided by the original sample weight. This value was then multiplied by 100 to derive the digestibility of dry matter percentage.

For protozoa counting, 1 mL of rumen fluid sample was mixed with 1 mL of a solution of 0.6 g methyl green, 8 g NaCl and 100 mL formaldehyde (37%) filled up to 1000 mL with distilled water. The portions of the samples were, then, pipetted into a counting chamber (Fuchs-Rosenthal: 0.0625 mm^2; 0.2 mm deep; Marienfeld, Germany). Total numbers of protozoa, without quantifying different types, were determined using a light microscope (Leica CME).

Statistical Analyses

Fermentation data were analyzed using two-way repeated measures analysis of variance (two-way ANOVA) with the SigmaStat 3.1 software (Systat Software, Erkrath, Germany). The model included the fixed effects of treatment, time, and their interaction and random effects of fermenters. The individual fermenter was used as the experimental unit for statistical analysis. In case of a significant ANOVA result, post hoc Duncan tests were performed to evaluate the statistical differences between the groups. P values of <0.05 were considered significant.

RESULTS

Effects of cleavers and yarrow extract on *in vitro* ruminal fermentation are given in *Table 4*. Neither cleavers nor yarrow extracts had a significant effect on ruminal pH, total VFA, acetate, propionate and methane production, NH_3-N concentration and, total protozoa. However, both extracts decreased dry matter digestibility (P<0.05). Butyrate production, on the other hand, increased with cleavers extract (P<0.05). Treatment × Time interaction was significant for total protozoa (P=0.004) and NH_3-N concentration (P=0.021). Total protozoa were higher (P<0.05) in the presence of yarrow and cleavers extracts than control on the 1st day of collection period and, on the 1st and 2nd days of the collection period, respectively. Cleavers and yarrow extracts also increased (P<0.05) NH_3-N concentration on the 4th day of the collection period.

DISCUSSION

In the present study, cleavers and yarrow extracts were evaluated for their potential application as modifiers of rumen microbial fermentation. Until recently, no studies have been conducted on the effects of cleavers on rumen microbial fermentation and, there is only limited data about the effects of yarrow on rumen fermentation. The extracts tested in this study showed no significant influence on the culture pH in RUSITEC fermenters. The pH values were within the normal range (6.5-7.0). Published reports on the effect of plant extracts on ruminal pH are variable. The findings of the present study for ruminal pH were agreed with previous researches that found no effect on ruminal pH [27-29]. The diet supplemented with a medicinal plant mixture containing yarrow as a component also did not change the pH of the sheep rumen [30,31].

Table 4. *Effects of cleavers (G. aparine) and yarrow (A. millefolium) extracts (500 mg/L) on ruminal fermentation in RUSITEC*

Parameters	Treatments			SEM	P Value
	Control	Cleavers Extract	Yarrow Extract		T × T
Ruminal pH	6.72	6.74	6.73	0.00	0.818
Total VFA (mmol/d)	27.54	27.93	27.87	1.10	0.861
Acetate	15.75	15.28	15.53	0.64	0.910
Propionate	8.29	8.50	8.56	0.43	0.869
Butyrate	3.51^{b}	4.15^{a}	3.78^{ab}	0.12	0.471
Methane (mmol/d)	7.55	7.60	7.51	0.28	0.870
DMD (%)	56.20^{a}	51.87^{b}	52.05^{b}	0.92	0.156
Protozoa (×10^3/mL)	5.67	10.71	8.63	1.64	0.004
NH_3-N (mmol/L)	6.02	6.36	6.03	0.18	0.021

^{a,b} Means in the same row followed by different superscripts differ significantly (P<0.05). VFA: Volatile fatty acids; DMD: Dry matter digestibility; T × T: Treatment × Time interaction.

Effects of plant extracts on ruminal fermentation are desirable if they increase or do not change total VFA production while the reduced VFA production is generally considered as a sign of depressed microbial fermentation [32]. The production of total VFA was not affected by both plant extracts in the present study. Our result is consistent with the report by Broudiscou et al.[33] in which *A. millefolium* (yarrow) extract at 500 mg/day did not affect the concentration of total VFA in 1 L dual outflow fermenters. Yarrow essential oil also did not change *in vitro* total VFA concentration in the dose range of 0-750 mg/L [34]. Yarrow extract did not change VFA profile in our study although it was reported to increase the molar proportion of acetate to the detriment of butyrate [33]. The conflicting results about VFA profile may be due to differences in the chemical composition of the diets and extracts used in different studies. On the other hand, the increase in butyrate production with the supplementation of cleavers extract in the present study suggests that the cleavers extract favored butyrate-producing bacteria at the used dose. Butyrate-producing bacteria in the rumen are generally in Gram-positive nature [35]. Cleavers extract used in the present study contained several phenolic compounds such as *p*-hydroxy benzoic acid, caffeic acid, benzoic acid, vanillin, and apigenin *(Table 1)*. There are reports about stimulatory effects of phenolic acids and flavonoids on growth and fermentation activity of particularly Gram-positive rumen bacteria [36-38].

The main significant findings of this study were the effects of cleavers and yarrow extracts on dry matter digestibility. Both extracts decreased digestibility of feed dry matter after 48 h incubation in RUSITEC. Broudiscou et al.[18] reported that *A. millefolium* (yarrow) extract depressed moderately *in vitro* organic matter digestibility as well as increasing degradability of crude protein and cell-wall constituents. *In vitro* degradability of dry matter and organic matter decreased by 500, 750 and, 1000 mg/L of yarrow essential oil according to the report by Kahvand and Malecky [34] which was consistent with our findings. However, they did not observe any adverse effect on digestibility at 250 mg/L dose. In the present study, yarrow extract at the used dose might also have inhibitory effects on some cellulolytic bacteria through the phenolic compounds it contained. Nevertheless, surprisingly no inhibition was observed for daily production of VFA. The decrease in dry matter digestibility without affecting the total VFA production may be related to a higher sensitivity and/or resistance of some specific type of cellulolytic bacteria [39]. According to another report, *in vitro* dry matter digestibility was lowest for yarrow after 24 h ruminal incubation [40]. The authors attributed this effect to the lower nitrogen content in comparison with the other examined herbs. Moreover, Hammond et al.[41] also reported that dairy heifers fed with a ryegrass and wild flower mixture of predominately sorrel, ox-eye daisy, yarrow, knapweed, and ribwort plantain had 18% lower DMD compared to control heifers fed with only ryegrass.

Methane production and NH_3-N concentration were also not affected by plant extracts. Compatible with the results of the present study, Broudiscou et al.[33] reported that *A. millefolium* (yarrow) extract at 500 mg/L dose did not have a significant effect on *in vitro* methanogenesis. Yarrow essential oil also did not mitigate *in vitro* ruminal methane production at 0-750 mg/L doses [34]. However, it lowered methane production at 1000 mg/L dose which was accompanied with a concomitant decrease in production of total gas and VFA, microbial biomass, and digestibility of dry matter as well as organic matter indicating a general inhibition of rumen fermentation [34]. The effects of plant extracts on rumen NH_3-N concentration are also dose-dependent and these compounds are more effective when used at high doses compared with at low doses. For example, Castillejos et al.[42] evaluated the effects of increasing doses of vanillin and eugenol on rumen fermentation in a 24 h *in vitro* fermentation and showed that these essential oils decreased NH_3-N concentration at the highest dose (5000 mg/L), but no effects were observed at lower doses.

The numbers of large protozoa were increased by *A. millefolium* (yarrow) extract in dual outflow fermenters according to Broudiscou et al.[33]. However, our extracts had no effect on rumen fluid protozoal counts except the increase in the first few days of the experiment. Type and amount of active components in the extracts might play a role in the expression of stimulatory or inhibitory effects against protozoa.

The present study showed that there were only small effects of cleavers and yarrow extracts on the investigated microbial fermentation characteristics in the semi-continuous rumen simulation technique (RUSITEC). Nevertheless, it may be considered advantageous for feed conversion that plant extracts did not suppress ruminal fermentation in spite of decreasing dry matter digestibility. However, further *in vitro* studies should be conducted using higher doses of these extracts, and *in vivo* studies are needed to evaluate their efficacy as alternative feed additives in ruminant nutrition.

Conflict of Interests

The authors declare no competing interests.

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Research Article

A Molecular Investigation of Carbapenem Resistant Enterobacteriaceae and *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ Genes in Raw Milk [1]

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Abstract

The success of antibiotic treatment has been negatively affected due to developing and spreading antimicrobial resistance all over the world. The present study was carried out to reveal the presence of carbapenem resistant Enterobacteriaceae and *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes responsible for carbapenem resistance in raw milk and to contribute to transmission dynamics and molecular epidemiology of carbapenem resistance, as well as the potential public health risks of milk. In Turkey, there is not sufficient data on the presence and the potential risks posed by carbapenem resistance in animal origin foods. A total of different 427 raw milk samples were collected and subjected to phenotypic microbiological analysis and conventional and Sybergreen real-time PCR targeting *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes. In the phenotypic analyses, suspicious isolates were identified by Vitek-2 compact system and antibiotic resistance profiles were revealed. Two *Stenotrophomonas maltophilia* inherently resistant to carbapenems were detected in raw milk samples. Acquired carbapenem resistance and related genes were not found in any of the milk samples. The present study revealed that milk is not epidemiologically involved in the transmission of carbapenem resistance. In order to prevent the environmental distribution of antibiotic resistant microorganisms, control of antibiotics used in human and veterinary medicine should be maintained.

Keywords: *Carbapenem resistant Enterobacteriaceae, Carbapenemases, Raw milk*

Çiğ Sütlerde Karbapenem Dirençli Enterobacteriaceae ve *bla*$_{KPC}$, *bla*$_{NDM}$ ve *bla*$_{OXA-48}$ Gen Varlığının Moleküler Olarak İncelenmesi

Öz

Uygulanan antibiyotik tedavileri, antimikrobiyel dirençliliğin dünya genelinde şekillenmesi ve yayılım göstermesi nedeniyle olumsuz yönde etkilenmektedir. Bu çalışma, potansiyel halk sağlığı risklerinin ortaya konması yanısıra, çiğ sütteki karbapenem dirençli Enterobacteriaceae ve karbapenem dirençliliğinden sorumlu *bla*$_{KPC}$, *bla*$_{NDM}$ ve *bla*$_{OXA-48}$ genlerinin varlığını ortaya koymak ve karbapenem direncinin taşınma dinamikleri ve moleküler epidemiyolojisine katkıda bulunmak amacıyla gerçekleştirilmiştir. Türkiye'de hayvansal gıdalarda karbapenem dirençliliğin varlığı ve sebep olduğu potansiyel riskler hakkında yeterli veri bulunmamaktadır. Toplanan toplam 427 farklı çiğ süt örneği fenotipik mikrobiyolojik analizlere tabi tutuldu ve *bla*$_{KPC}$, *bla*$_{NDM}$ ve *bla*$_{OXA-48}$ genlerini hedef alan konvansiyonel ve Sybergreen real-time PCR işlemleri gerçekleştirildi. Fenotipik analizler sonucunda şüpheli izolatlar VITEK-2 kompakt sistem ile tanımlandı ve antibiyotik dirençlilik profilleri ortaya kondu. Çiğ süt örneklerinde karbapenem grubu antibiyotiklere doğal dirençli iki *Stenotrophomonas maltophilia* tespit edildi. İncelenen hiçbir süt örneğinde kazanılmış karbapenem direnci ve ilişkili genler tespit edilmedi. Bu çalışma, epidemiyolojik olarak sütün karbapenem dirençliliği dağılımında rol almadığını göstermektedir. Antibiyotiğe dirençli mikroorganizmaların çevresel dağılımını önlemek için, insan ve veteriner hekimlikte kullanılan antibiyotiklerin kontrolüne devam edilmelidir.

Anahtar sözcükler: *Karbapenem direneçli Enterobacteriaceae, Karbapenemazlar, Çiğ süt*

INTRODUCTION

Enterobacteriaceae is a family of rod-shaped, Gram negative strains naturally present in the intestinal biota of warm-blooded animals. It contains pathogens that cause severe diseases such as cystitis, pyelonephritis, septicemia, pneumonia, peritonitis and meningitis, and some Enterobacteriacea can cause foodborne disease.

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Bacteria belonging to this family can be easily distributed between humans and other warm-blooded organisms via food and water, and genetic elements can be transferred to each other through mobile structures such as plasmids and transposons [1-3]. Due to these properties, Enterobacteriaceae plays a significant role in contributing to multidrug resistance.

Carbapenems form an antibiotic group that is effectively used against the Gram-negative bacilli that develop broad-spectrum β-lactamase antibiotic resistance. Carbapenem resistance make it more difficult to treat nosocomial diseases [4]. Carbapenem resistant Enterobacteriaceae (CRE) is the current common problem reported worldwide [5-7].

The use of carbapenems in farm and pet animals has not been approved due to the clinical importance of these antibiotics and resistance concerns [8]. For this reason, carbapenem resistance can be predicted to be very rare in isolates of animal and related foods and there is limited data on the current state and prevalence of carbapenem-resistant bacteria in animals and their associated environments [9]. However, some studies conducted in different countries have recently shown that carbapenem resistance is observed apart from human isolates. It has been reported that carbapenem-resistant bacteria from various animals were detected in Germany [10], France [11], Belgium [12] and China [13]. For this reason, resistance to antibiotics used effectively in cases of serious infection should be monitored and reported globally.

The present study was conducted to reveal whether CRE could be distributed widely with raw milk which is consumed and processed in the dairy industry. An important public debate continues on the possible benefits of increasing the popularity of raw milk consumption [14]. In this matter, the aim of the study is to determine the role of animal originated foods in the epidemiology of CREs and the role of healthy individuals and animals in reaching the sensitive groups.

MATERIAL and METHODS

Bacterial Strains

Escherichia coli MSC234 (pMSC122); *E. coli* MSC229 (pMSC116), *E. coli* MSC228 (pMSC115) and *Klebsiella pneumoniae* ATCC BAA-1705 cultures were used as a positive control and *E. coli* ATCC 25922 were used as a negative control.

Sample Collection

A total of 427 milk samples from dairy cows were included in the study for a two-year period. Each sample was collected from separate cows from 15 different dairy farms located in Central Anatolia region, Turkey. A total of 40 mL milk samples, 10 mL from each nipple, were taken from the cows into sterile 50 mL falcon tubes and brought to the laboratory under the cold chain. Before sampling, the udder was cleaned with a commercially ready to use iodophor based antiseptic solutions and dried with a paper towel.

Isolation of CRE from the Raw Milk Samples

The Isolation of CRE from raw milk collected in the study was performed by modifying the laboratory protocol proposed by the Centers for Disease Control and Prevention [15]. Briefly, 100 µL homogenized milk samples were put into tubes containing 5 mL sterile Tryptic Soy Broth (Merck, Germany) with a Meropenem disk (10 µg, Oxoid, United Kingdom) and the tubes were incubated at 35±2°C for 24 h. After incubation, 100 µL of the homogenized sample was streaked on the MacConkey Agar (Merck, Germany) and incubated at 35±2°C for 24 h. Suspected colonies with different morphology grown on the MacConkey agar were streaked on Chromagar™ KPC (Chromagar, France) and ChromID® Carba Smart (Biomerieux, France) selective agars and incubated at 35±2°C for 24 h as an initial screening step. Isolates growth in both selective media were evaluated as CRE suspicious.

Determination of Carbapenem Resistance Profiles of Suspected Isolates

Suspicious colonies isolated from selective agars were subjected to the disc diffusion method, Modified Hodge tests (MHT) and Modified Carbapenemase Inactivation method (mCIM) according to CLSI [16,17].

Identification of Carbapenem Resistant Isolates

Phenotypically positive pure isolates were cultivated in blood agar, and identification and antibiotic resistance profiles were analyzed by Vitek® 2 Compact system (Biomerieux, France) following the instructions of the manufacturer. Automated selection of antibiotics was done according to the EUCAST [18].

Determination of bla_{KPC}, bla_{NDM} and bla_{OXA-48} Carbapenemase Genes

Genomic DNA Extraction: The total genomic DNA extraction was performed using the Instagene Genomic DNA Extraction Kit (Bio-Rad, USA) from the suspected colonies according to the manufacturer's protocol. Concentrations of the gDNA samples (µg/µL) were measured by Qubit 3.0 fluorometer (Thermo Fisher, USA) and stored at -20°C until molecular analyzes.

PCR Amplification: The gDNA isolates were subjected to PCR analysis with specific primers in order to amplify the gene regions sought in the suspicious samples. For the amplification of bla_{KPC}, bla_{NDM} and bla_{OXA-48} carbapenemase genes, the primers designed by Poirel et al.[19] were used in the study *(Table 1)*. The Maxima Hot Start Green 2x PCR Master Mix (Thermo Fisher, USA) was used for PCR

analysis according to the manufacturer's instructions. PCR amplification was performed with an initial denaturation of 95°C for 4 min followed by 35 cycles, each consisting of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min. The final extension cycle was performed at 72°C for 10 min (Arktic™ Thermal Cycler; Thermo Fisher, USA). All amplification products were analyzed by agarose gel (1.5%) electrophoresis at 100 V for 45 min. The gels were stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA) and visualized under a UV transilluminator (Vilber Lourmat, France).

SYBR Green Real-Time PCR Amplification: For the real time amplification of *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ carbapenemase genes, the primers designed by Subirats et al.[20] were used in the study *(Table 1)*. qPCR was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) on the CFX96 Connect Real-Time System (Bio-Rad, USA) according to manufacturer's instruction. The cycling protocol consisted of 95°C for 3 min, followed by 40 cycles at 95°C for 30 s and 60°C for 45 s. A melting curve was constructed in the range of 60 to 95°C to verify the specificity of the amplified products in all analyses. Each sample was run with duplicate. The positivity and quantitative values in the samples were based on the amplification curves and Ct (dR) (Threshold value cycle) data and the melting curve profiles.

RESULTS

Isolation of CRE from the Raw Milk Samples

In the study, 93 different carbapenem resistant suspected isolates were collected with CDC protocols in conventionally examined raw milk samples. After cultivation on chromogenic agars of isolates obtained by CDC protocol, growth was observed in 52 isolates. In the disc diffusion, two of 52 suspected isolates were found to be meropenem resistant and none of the isolates were found to be positive in the mCIM and MHT tests.

Determination of Carbapenem Resistance Profiles of Suspected Isolates

As a result of phenotypic antimicrobial susceptibility tests, two carbapenem resistant isolates were identified as *Stenotrophomonas maltophilia* with the Vitek-2 Compact system. In the antimicrobial resistance, both isolates were found to be susceptible to Trimethoprim-sulfamethoxazole.

Determination of bla$_{KPC}$, bla$_{NDM}$ and bla$_{OXA-48}$ Carbapenemase Genes

According to the PCR and qPCR analyses, no *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ carbapenemase genes were detected in any gDNA obtained from the 52 carbapenem resistant suspected isolates collected from raw milk. Gel electrophoresis results obtained by PCR and Sybergreen qPCR results of *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ positive controls were shown in *Fig. 1* and *Fig. 2*, respectively.

DISCUSSION

Farm animals are reservoirs for many zoonotic pathogens, also may pose serious hazards to public health if they provide antibiotic resistance to these microorganisms. Particularly, the antibiotic resistance genes can be transferred to other bacteria and mutated to reveal the necessity to focus on animal foods more carefully regarding public health [21].

Nowadays, the control of antibiotic resistance is becoming more difficult. In the absence of a systematic route in the control of bacterial pathogens, the misuse and overuse of antibiotics lead to the development of resistant pathogens. The environmental microbiota has a wide variety of distribution in farms and food industries where animal food is obtained. It is known that mobile genetic elements can be easily shared in these complex circumstances. Animal food is easily contaminated with these pathogens and can reach the consumer as a result of the production of raw

Table 1. *Primer pairs used in this study*

Target Gene	Primer	Sequence (5'-3')	Product (bp)	Reference
bla$_{KPC}$	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	798	[19]
	KPC-Rm	CTTGTCATCCTTGTTAGGCG		
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621	
	NDM-R	CGGAATGGCTCATCACGATC		
bla$_{OXA-48}$	OXA-F	GCGTGGTTAAGGATGAACAC	438	
	OXA-R	CATCAAGTTCAACCCAACCG		
bla$_{KPC}$ allels	Kpc-rtF	CAGCTCATTCAAGGGCTTTC	196	[20]
	Kpc-rtR	GGCGGCGTTATCACTGTATT		
bla$_{NDM}$ allels	Ndm-rtF	GATTGCGACTTATGCCAATG	189	
	Ndm-rtR	TCGATCCCAACGGTGATATT		
bla$_{OXA-48}$ allels	Oxa-rtF	AGGCACGTATGAGCAAGATG	189	
	Oxa-rtR	TGGCTTGTTTGACAATACGC		

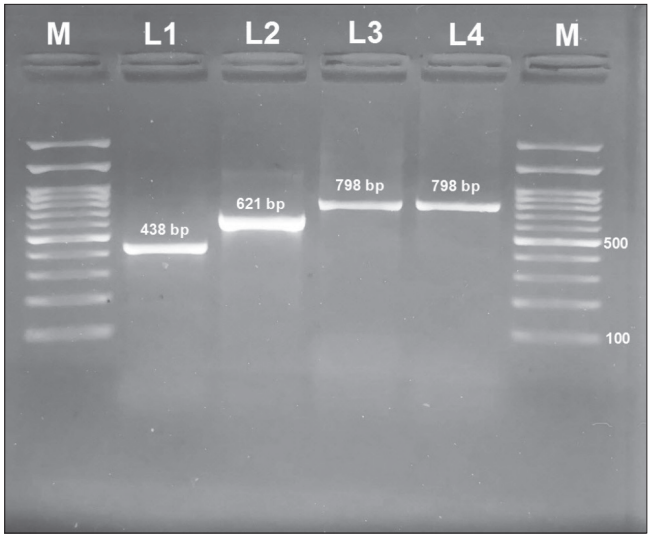

Fig 1. Agarose gel electrophoresis results of PCR assay for the bla_{KPC}, bla_{NDM} and bla_{OXA-48}. M: Marker (Geneaid 100-bp DNA Ladder); L1: Positive control for bla_{OXA-48} (*E. coli* MSC234 [pMSC122]); L2: Positive control for bla_{NDM} (*E. coli* MSC229 [pMSC116]); L3-4: Positive control for bla_{KPC} (*E. coli* MSC228 [pMSC115], *K. pneumoniae* ATCC® BAA-1705, respectively)

the spread of an antimicrobial resistance situation outside of the hospitals. The actual prevalence of carbapenem resistance is not yet known exactly and global scale studies on this subject are ongoing. In a report published in 2015 by the European Centre for Disease Prevention and Control (ECDC), it is stated that OXA-48 enzyme is endemic, NDM shows regional spread and KPC has not been reported in Turkey [27].

In the present study, CRE was not detected in any milk samples collected from different dairy farms in Central Anatolia, Turkey. However, in many recent studies, raw milk and dairy cows were reported to be positive for CRE in different countries. In a study conducted in Lebanon, it was reported that 30.2% CTX-M-15-producing *K. pneumoniae* was detected in raw bovine milk [28]. In another study conducted in India, it was reported that bla_{NDM-5} carbapenemase gene was found in one *E. coli* isolated from milk samples obtained from mastitic cow [29]. In a study organized in China, it was reported that 10 carbapenem resistant *K. pneumonie* were isolated in 65 milk and fecal samples from three dairy farms [30]. In a study

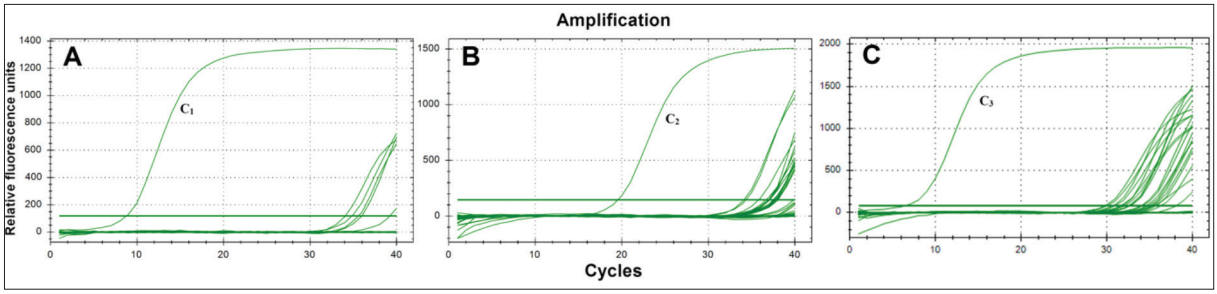

Fig 2. Sybergreen qPCR results of bla_{KPC}, bla_{NDM} and bla_{OXA-48}. **A:** Positive control for bla_{OXA-48} (*E. coli* MSC234 [pMSC122]); **B:** Positive control for bla_{NDM} (*E. coli* MSC229 [pMSC116]); **C:** Positive control for bla_{KPC} (*K. pneumoniae* ATCC® BAA-1705)

materials from non-hygienic farms and food production facilities with insufficient sanitation [22]. Inadequate farm conditions, operational errors such as faulty pasteurization, and personnel related contamination are important in terms of raw milk safety. The use of β-lactams has been limited by the wide spread of resistant *E. coli* strains containing broad-spectrum b-lactamase (ESBL) [23]. In consequence of this situation, the use of carbapenems globally increased due to their resistance to broad-spectrum β-lactamase hydrolysis from bacterial plasmids or chromosomes [24]. Enterobacteriaceae carrying NDM enzyme are resistant to almost all antibiotics with the few exceptions such as colistin. The presence and sharing of this plasmid mediated gene among bacteria originated from animal sources and food chain may pose a serious threat to human health [25].

Carbapenems are frequently used in the treatment of ESBL involved infections and play an important role in the treatment of nosocomial diseases [26]. The development of carbapenem resistance causes serious problems in the use of antibiotics and in the treatment of persistent infections. It is necessary to vary epidemiologic studies to determine

in Algeria, four *E. coli* isolates with identical PFGE profiles were obtained from the raw milk and teats of 34 healthy cows and carbapenem resistance was detected in all of *E. coli* isolates [31]. In a study carried out in Brazil, it is revealed that none of the 117 *E. coli* exhibited carbapenemase activity isolated from raw milk used in cheese production without any heat treatment, as a similar finding to current study [32]. In regard to these data, the main objective of this study was to determine the occurrence of CRE and carbapenemase in raw milk, in order to help clarify risk assessment with regard to potential human transfer through milk consumption. In this regard, it should be stated that there are no concerning levels of CRE in farm animals and related food products in Turkey.

In the findings of the present study, it is revealed that determination based on agar selection is not sufficient. It is observed that chromogenic agars have not exhibited essential sensitivity in the detection as claimed. None of the 52 suspected isolates obtained from selective agar had carbapenem resistance. Also, in the phenotype-based susceptibility methods, there are inherent problems in

differentiation between acquired and intrinsic resistance [33]. The isolates that were found to be cabapenem resistant in the study were detected as *S. maltophilia* by the VITEK compact system. For this reason, it is thought that the most accurate antimicrobial resistance determination can be realized by DNA based molecular methods. *S. maltophilia* is a Gram-negative bacillus showing wide environmental spread and although of low virulence pathogenicity, may cause serious infections in debilitated or immune-compromised individuals and patients. This environmental bacterium is known to have intrinsic resistance to many antibiotics with properties such as efflux pumps and low membrane permeability [34]. In the study, both of *S. maltophilia* inherently resistant to carbapenems were found to be susceptible to Trimethoprim-sulfamethoxazole. It is shown that monitoring of environmental contamination and control measures should be considered in animal food.

The results of the study showed that milk is not involved in the transmission of carbapenem resistance and the epidemiological data obtained are significant. CREs were not detected in any of the 427 milk samples and the resistance of these group antibiotics prohibited in the veterinary field has not developed in the dairy environment. Distribution of environmental microorganisms plays an important role in the continuation of the effectiveness of antibiotics such as carbapenems considered to be last resort antibiotics in the treatment of persistent nosocomial infections. Pathways by which bacteria and antimicrobial resistance instruments spread to humans from farm to fork should be clearly understood.

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Research Article

Evaluation of Serum Amyloid A and Procalcitonin in Some Inflammatory Diseases of Cattle

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Abstract

Recent study in humans and animals has been focused on inflammatory biomarkers that infectious diseases, such as serum amyloid A (SAA), procalcitonin (PCT), that may more accurately and efficiently diagnose inflammation. The aim of this study was to evaluate SAA and PCT levels in the diagnosis of cattle with inflammatory disease. Ten healthy control cattle and 64 patients with systemic inflammatory response syndrome (SIRS) were included in cattle. Inflammatory disease in cattle was diagnosed based on clinical signs and the laboratory examination in clinically suspected cases. SAA and PCT concentrations were measured with a commercial ELISA assay for cattle. SAA and PCT concentrations in cattle with inflammatory disease were significantly higher than in the healthy controls (respectively, $P<0.001$, $P<0.008$). Concentrations of SAA and PCT at admission were significantly ($r = 0.376$, $P<0.01$) correlated with outcome in cattle with inflammatory conditions. The cut-off value of SAA and PCT for healthy and inflammatory cattle was determined 28.52 µg/mL and 149.55 pg/mL. In conclusion, PCT levels may be used as an alternative to serum SAA measurement as an indicator of acute inflammation in cattle. Serum PCT concentrations were ~9 times higher in the cattle with peritonitis than in the healthy cattle, suggesting that PCT could be a useful marker of peritonitis in cattle.

Keywords: *Cattle, Marker, Procalcitonin, Serum amyloid A*

Sığırların Bazı İnflamatuar Hastalıklarında Serum Amiloid A ve Prokalsitoninin Değerlendirilmesi

Öz

İnsanlarda ve hayvanlarda yapılan son çalışmalar, enfeksiyöz hastalıkları daha doğru ve etkili şekilde teşhis edebilmek için serum amiloid A (SAA) ve prokalsitonin (PCT) gibi infamasyon biyobeliteçlerine odaklanmıştır. Bu çalışmanın amacı, inflamatuar hastalığı olan sığırların tanısında SAA ve PCT seviyelerinin değerlendirilmesidir. On sağlıklı kontrol sığır ve sistemik inflamatuar yanıt sendromlu (SIRS) 64 hasta sığır dahil edildi. Sığırlarda inflamatuar hastalık klinik olarak şüpheli vakalarda, klinik bulgular ve laboratuvar incelemelerine dayanarak teşhis edildi. SAA ve PCT konsantrasyonları, sığırlar için ticari bir ELISA ile ölçüldü. Enflamatuar hastalığı olan sığırlarda SAA ve PCT konsantrasyonları sağlıklı kontrollerden anlamlı olarak daha yüksekti (sırasıyla, $P<0.001$, $P<0.008$). SAA ve PCT'nin başvuru sırasındaki konsantrasyonları, enflamatuar koşulları olan sığırlarda, sonuçlar anlamlı derecede ($r = 0.376$, $P<0.01$) ilişkiliydi. Sağlıklı ve inflamatuar hastalıklı sığırlar için SAA ve PCT'nin cut-off değeri 28.52 µg/mL ve 149.55 pg/mL olarak belirlendi. Sonuç olarak; PCT düzeyleri, sığırlarda akut enflamasyonun bir göstergesi olarak serum SAA ölçümüne alternatif olarak kullanılabilir. Serum PCT konsantrasyonları, peritonitisli sığırlarda sağlıklı sığırlara göre yaklaşık 9 kat daha yüksekti; bu, PCT'nin sığırlarda faydalı bir peritonit belirteci olabileceğini düşündürmektedir.

Anahtar sözcükler: *Sığır, Belirteç, Prokalsitonin, Serum amiloid A*

INTRODUCTION

One of the most important aspects of the complete evaluation and follow-up of the diseases in the veterinary medicine is to determine the inflammatory condition. Early detection of systemic inflammatory status is indispensable for an effective treatment plan [1]. Infections and inflammatory events cause a systemic response in the organism, which is

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called the acute phase response (APR). APR is a natural defense mechanism that stimulates healing following trauma, infection, or inflammation for limiting tissue damage [2,3]. APR ensures the initiation of repair process which is necessary; for preventing further damage to the organs, for isolation and destruction of infectious agents, for removal of harmful molecules and residues and for the organs to regain its functions. This response of the organism also includes changes in plasma protein concentrations, which are synthesized by the liver and called its APRs [1,2]. Different inflammatory molecules introduced into circulation with the effects of immune system mechanism against infection in organism. These molecules are thought to be diagnostic markers and can be used to monitor infections [4]. Serum amyloid A (SAA), which is an important acute phase protein in cattle, is one of the markers used for the diagnosis of infections, the presence of bacteremia, the course of the disease and mortality [5,6]. SAA, which is used as markers of inflammation or infection, has been used in many studies in this context. SAA is an acute phase protein produced by the liver. The plasma concentrations of SAA are normally very low, but it has been reported that the blood values increase after trauma, inflammation and tissue damage. Especially infectious agents lead to a sudden rise in SAA values [1,2,4].

Procalcitonin (PCT) is a prohormone of calcitonin and is a 116 amino acid protein with a molecular mass of 13 kDa [7]. In a normal metabolic state, hormonal active calcitonin is produced and secreted by C-cells of the thyroid gland after proteolytic treatment processing of PCT intracellularly. It has been reported that in infective cases PCT is secreted from the extrathyroidal source such as liver and lung [8-10]. The mediators produced in response to endotoxins or bacterial infections are said to induce the production of PCT. It was reported that PCT started to increase 4 h after inflammation, peaked about 6 h later, and rapidly returned to normal values after the inflammation was controlled by the organism [11]. Serum PCT level is associated with the prevalence and severity of bacterial infections. It has also been reported that increased interferon gamma (INF-γ) during viral infections suppresses PCT production and may be useful in differentiating viral and bacterial infections [12,13]. It has also been stated that PCT levels above 0.5 ng/mL may be an indicator of diseases in human medicine [14].

This study examines the levels of SAA, which has recently become a common practice for following up infections, and PCT, which is recently used in veterinary medicine, in some important diseases in cattle, and the correlations between them.

MATERIAL and METHODS

Ethics

This study was approved by the Local Ethics Committee for Animal Experiments (Approval number: 2017/117), Sivas Cumhuriyet University.

Animals

The study animals consisted of 74 cattle referred to Sivas Cumhuriyet University Veterinary Teaching Hospital (Sivas, Turkey) for various diseases. In the study, there were the Holstein (n=12), the Simmental (n=32), and the Swiss Brown (n=20) cattles, different sex (52 females, 12 males), ranging in age from 90 day to 3 years. The study included acute diseased animals. Septic patients were not included in the study. Among the diseases included in the study, respiratory (pneumonia, 10), reproductive systems (retentio secundinarum, 6; metritis, n=10), mastitis 10, omphalitis (n=10), arthritis (n=10), peritonitis (n=8), control (n=10). Similarly, the cattle included in the control group were also of different sex (8 females, 2 males) and breed (2 Holstein, 5 Brown Swiss, and 3 Simmental cattle), age (90 days-3 years).

After clinical examinations, blood samples were collected from the *v. jugularis* to sterile test tubes for laboratory analysis. After centrifugation of test tubes at 3000 rpm for 15 min, serum samples were stored at -80°C for evaluation SAA and PCT.

SAA Measurement

The SAA concentration was measured by the solid phase sandwich ELISA method (Tri-Delta Phase SAA, Tri-Delta Diagnostic, Boonton Township, NJ). Samples were analyzed by diluting 1/500. The serum or plasma analytical sensitivity of this test in cattle was determined as 1.5 ug/mL by the manufacturer. The intra and inter assay precision-reproducibility (CV%) of the test was 7.5% and 12.1% for cattle, respectively.

PCT Measurement

Serum PCT concentration was determined by ELISA method using a commercial kit (Bovine procalsitonin ELISA Kit, SunRed, Ltd. Shanghai, China). This ELISA kit is based on the principle of double-antibody sandwich technique to detect bovine. The analytical sensitivity of this test in cattle was determined as 8.775 ng/mL by the manufacturer.

Statistical Analysis

Statistical analyses were performed using the 15.0 SPSS package programme (Statistical Package for Social Sciences, Chicago, IL). The variables were tested for normal distribution with the Kolmogorov-Smirnov test. Comparisons between groups were made by use of non-normal distribution were analysed with the Mann-Whitney U test. Furthermore, correlations between SAA and PCT levels were assessed with Spearman's correlation coefficients. Receiver operating characteristic (ROC) curves and the area under these curves were used to assess the diagnostic potential of SAA

and PCT levels. ROC analyses were performed for both the healthy and cattle with inflammatory disease. To assess the diagnostic potential of SAA and PCT levels in the diagnosis of the cattle with inflammatory disease, the area under curve (AUC) and some cut-off values were analyzed. The results were assessed at a 95% confidence interval and at a significance level of P<0.05.

RESULTS

A total of 74 cattle, including healthy (n=10) and sick cattle (n=64), were included to the study. Clinically, general findings such as increased body temperature, fatigue, loss of appetite, reduction in mobilization, lameness, pain in the flexion of the relevant joint, local temperature increase, swelling and sensitivity at varying levels were identified in all arthritis calves. The calves with omphalitis had swelling and tenderness in the umbilical cord, increased body temperature and weakened suction reflex. Mastitis was diagnosed with clinical examination and California Mastitis Test. Animals without systemic clinical symptoms and with a purulent or mucopurulent discharge from the uterus were evaluated with clinical endometritis 21 days or more after birth. Increased respiratory rate, tracheal tenderness, cough and nasal discharge and high fever were observed in the pneumonia. At the 12th h following the delivery, those who were not able to expel some or all of the offspring placenta were considered as retentio secundinarum. In diagnosis of peritonitis, reduction in cattle appetite, increase in body temperature in heart rate, respiration and ultrasonography was used for diagnosis of peritonitis. Pericarditis traumatica not included.

Table 1. *Serum amyloid A and procalcitonin determined in diseases and control cattle*

Parameters	Control Group (n=10; mean±SE)	Diseased Group (n=64; mean±SE)	P Value
SAA (µg/mL)	21.66±9.40	152.84±9.19	0.001
PCT (pg/mL)	149.84±65.71	352.41±65.71	0.008

SAA: *Serum amyloid A;* ***PCT:*** *Procalcitonin*

Table 2. *Serum serum amyloid A and procalcitonin values of diseases and control animals*

Disease	SAA (µg/mL) (mean±SE)	PCT (pg/mL) (mean±SE)
Arthritis (n=10)	194.76±8.99	151.93±12.44
Omphalitis (n=10)	160.21±20.96	273.54±45.50
Mastitis (n=10)	83.36±28.63	160.25±18.05
Metritis (n=10)	118.95±24.61	207.15±21.30
Pneumonia (n=10)	160.62±14.66	231.47±32.82
Retentio secundinarum (n=6)	205.72±5.61	430.47±134.89
Peritonitis (n=8)	171.07±29.00	1215.97±407.53
Control (n=10)	12.49±2.35	139.26±12.36

SAA: *Serum amyloid A;* ***PCT:*** *Procalcitonin*

When SAA and PCT levels of healthy and sick cattle were compared, statistically significant difference (P<0.001) was found *(Table 1)*. Cattle in the patient group were grouped according to their disease. The grouping is shown in *Table 2*. Cattle were divided into groups according to their diseases and their importance levels among themselves and healthy cattle were show in *Table 3*. While the highest significance values (P<0.001) were found in arthritis group and omphalitis group in SAA, it was found that P<0.001 level in peritonitis group according to PCT levels. A

Table 3. *Importance between sick and healthy animals of serum amyloid A and procalcitonin*

Diseases	SAA (µg/mL)	PCT (pg/mL)
Arthritis - Omphalitis	0.290	0.041
Arthritis - Mastitis	0.013	0.094
Arthritis - Metritis	0.041	0.041
Arthritis - Pneumonia	0.096	0.016
Arthritis - Retentio secundinarum	0.745	0.005
Arthritis - Peritonitis	0.722	0.000
Omphalitis - Mastitis	0.034	0.034
Omphalitis - Metritis	0.226	0.406
Omphalitis - Pneumonia	0.597	0.650
Omphalitis - Retentio secundinarum	0.278	0.233
Omphalitis - Peritonitis	0.722	0.026
Mastitis - Metritis	0.406	0.07
Mastitis - Pneumonia	0.059	0.041
Mastitis - Retentio secundinarum	0.03	0.007
Mastitis - Peritonitis	0.076	0.001
Metritis - Pneumonia	0.151	0.705
Metritis - Retentio secundinarum	0.051	0.083
Metritis - Peritonitis	0.155	0.003
Pneumonia - Retentio secundinarum	0.039	0.193
Pneumonia - Peritonitis	0.214	0.003
Retentio secundinarum - Peritonitis	0.796	0.121
Arthritis - Control	0.000	0.327
Omphalitis - Control	0.000	0.011
Mastitis - Control	0.41	0.41
Metritis - Control	0.003	0.018
Pneumonia - Control	0.000	0.003
Retentio secundinarum - Control	0.000	0.002
Peritonitis - Control	0.009	0.000

SAA: *Serum amyloid A;* ***PCT:*** *Procalcitonin*

Table 4. *Correlations between serum amyloid A and procalcitonin*

Parameters	SAA (µg/mL)	PCT (pg/mL)
SAA (µg/mL)	-	0.376**
PCT (pg/mL)	0.376**	-

*** P<0.01,* ***SAA:*** *Serum amyloid A,* ***PCT:*** *Procalcitonin*

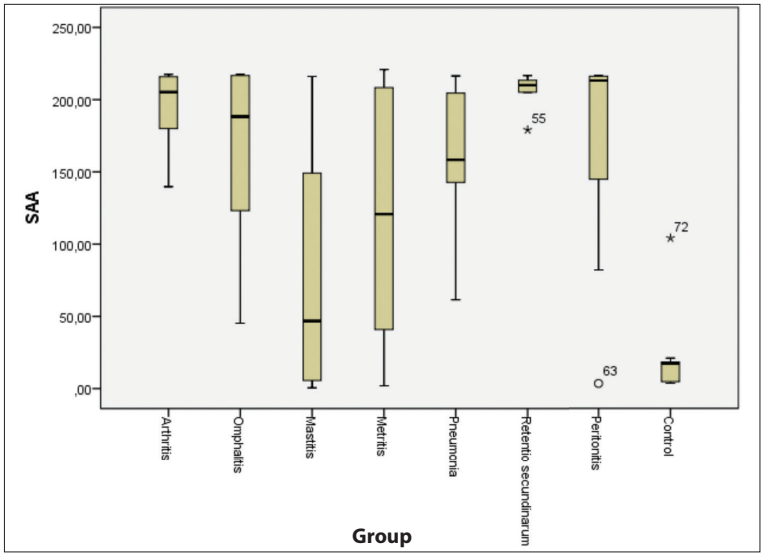

Fig 1. Distribution of serum amyloid A (SAA) among diseased and control cattle

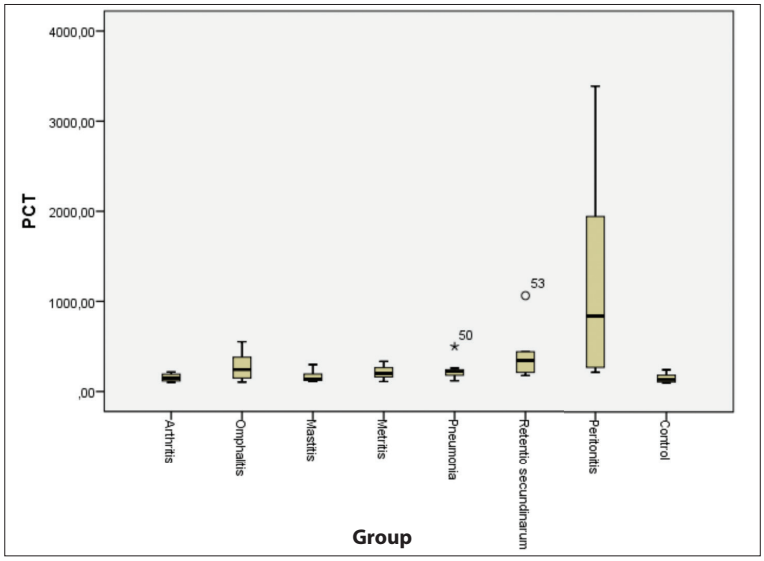

Fig 2. Distribution of procalcitonin (PCT) among diseased and control cattle

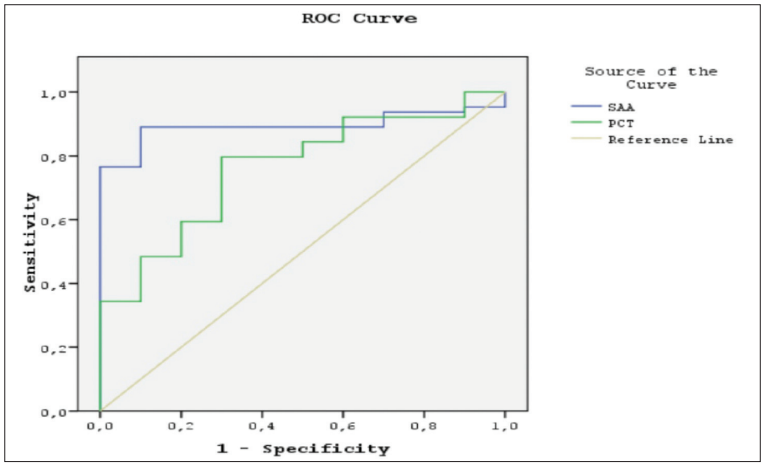

Fig 3. The receiver operating characteristic (ROC) curves used to assess the diagnostic potential of SAA and PCT levels

significant correlation was found between SAA and PCT levels of all cattle ($r = 0.376$, $P<0.01$) *(Table 4)*. The distribution between SAA and PCT is shown in the *Fig. 1* and *Fig. 2*.

Receiver Operating Characteristic (ROC) analysis was conducted on the SAA and PCT levels in the healthy and cattle with inflammatory disease. The ROC curves used to assess the diagnostic potential of SAA and PCT levels, are presented in *Fig. 3*. *Table 5* shows the cut-off, sensitivity, specificity, the areas under the curves (AUC) of the SAA and PCT levels of the cattle with inflammatory disease. The cut-off values for the SAA and PCT levels were determined as 28.52 µg/mL and 149.55 pg/mL respectively.

DISCUSSION

Diseases associated with inflammation such as arthritis, metritis, omphalitis, pneumonia and peritonitis may cause significant economic losses or deaths in cattle. In veterinary medicine, the tests used to determine the presence of inflammation in the acute inflammation of cattle are valuable in terms of its contribution to the determination of clinic, diagnosis and prognosis of diseases [2,6,15,16].

Glycoproteins whose blood concentrations change rapidly after tissue damage are defined as APP, and the resulting response is called as APR [1,2]. It is considered to be the first condition for maintaining physiological homeostasis and sustaining life after tissue damage. APR is a part of the nonspecific immune response, and some components vary due to the wide distribution of stimulatory conditions. The synthesis of these proteins, whose concentrations vary positively or negatively, usually occurs in the liver. In this context, acute phase proteins have been used in veterinary clinical biochemistry as nonspecific variables for monitoring inflammation activity [4,17].

It is stated that the clinical value of APPs varies according to animal, species and this should be taken into account in the evaluation of APPs. In particular, SAA is considered to be one of the most important APPs for cattle because there are low levels of SAA in healthy cattle, yet there are high levels of SAA in blood during acute phase response [6,18]. Therefore, the level of SAA was determined in order to assess significance of the value in PCT levels.

Serum amyloid A frequently preferred as inflammatory marker on different clinical pathologies and patient groups in recent years.

Table 5. *The cut-off, sensitivity, specificity, the areas under the curves values of the serum amyloid A and procalcitonin levels in diseases and control cattle*

Parameters	Cut-off	Sensitivity (%)	Specificity (%)	AUC	P Value
SAA (µg/mL)	28.58	89	90	0.894	0.000
PCT (pg/mL)	149.55	70	70	0.763	0.008

SAA: *Serum amyloid A;* ***PCT:*** *Procalcitonin;* ***AUC:*** *The areas under the curves*

SAA is from the apolipoprotein family of high density lipoprotein. It is an acute phase protein expressed at different levels in inflammatory reactions [1]. SAA is a rapidly reacting APP, which shows a high level of inflammation in cattle. For example, it has been stated that the mean of SAA levels in cattle with acute diffuse peritonitis is 312.4 µg/mL, and that a statistically significant increase can be useful in clinical medicine [19]. Especially in the diagnosis of mastitis, the determination of SAA levels in blood serum and milk has been reported to be useful in identifying acute, chronic and subclinical conditions of the disease, as well as in distinguishing between mild and moderate conditions [20,21].

In this study, SAA levels in healthy cattle are consistent with previous studies. The 4-9 fold increase in SAA in cows with arthritis, pneumonia, mastitis, retained placenta compared with healthy cattle, were similar in magnitude to that reported previously in cows with traumatic reticuloperitonitis, mastitis, metritis, pododermatitis, and abdominal infection [22,23].

The significance level for the comparison between study group and healthy cattle was determined to be P<0.001. In addition, the highest increases in SAA levels were observed in patients with retentio secundinarum, arthritis, peritonitis and pneumonia, while the largest statistical significance was found in arthritis, omphalitis and pneumonia groups when compared with healthy cattle (P<0.001). In addition, these results suggest that clinical use of SAA may be beneficial in inflammatory disease in cattle

Procalcitonin is an acute phase reactant which has been studied frequently in medicine in recent years. Viral diseases and autoimmune diseases do not cause increases in PCT. Therefore, in human medicine PCT is most commonly used to distinguish between bacterial diseases and non-bacterial diseases [24,25]. In addition, serum PCT levels were found to be high in sepsis, bacteremia, meningitis and fungal infections causing serious systemic infection [24,26,27]. This observation is consistent with the findings of previous researches and may be explained by the fact that IFN-γ inhibits IL-1 beta-induced calcitonin mRNA expression and PCT secretion [12,13,28]. All values of PCT above 0.5 ng/mL are considered important and indicate that patients are in life-threatening conditions [9]. In addition, it has been reported that it is a significant advantage that PCT has a long serum half-life and is stable when kept at room temperature [28].

Veterinary research has reported that PCT has shown significant increases in septic calf infections [28,29]. No study on the diagnosis of large-scale inflammatory diseases has been found as a result of literature review although previous studies in human medicine have also used C reactive protein (CRP) and PCT level to identify of some inflammatory diseases [24-26].

In this study, the difference between SAA and PCT levels of healthy and infected cattle was determined as significant (P<0.001). It is noteworthy that PCT is higher when compared to SAA in patients with the diagnosis of peritonitis. Therefore, it can be concluded that PCT is more suitable for use in the diagnosis of peritonitis than SAA. In addition, a significant correlation between SAA and PCT indicates that PCT can be a valuable marker of infection. The study is supported by another recent study of neonatal calves with septicemic colibacillosis, which concluded that PCT concentrations were significantly higher, when compared to healthy subjects, and that a positive correlation was found between PCT and proinflammatory cytokines [28-30].

In conclusion, it was determined that SAA and PCT concentrations were significantly increased in the acute phase of the disease in cattle with inflammatory infection and there was a positive correlation between them. In addition, it was concluded that further studies on animals should be carried out to reveal the clinical significance of PCT.

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Research Article

Expression Profiles of Toll-like Receptors 2, 7 and 8 in Rat Testis and Epididymis During Postnatal Developmental Period [1]

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Abstract

Toll-like receptors take an essential part in innate immunity in response to invasion of the various harmful pathogens. We aimed to investigate TLR2, 7 and 8 expression in rat testis and epididymis throughout postnatal development. In the prepubertal period, TLR2 and 7 were variably localized to peritubular myoid cells, interstitial cells, blood vessels, epithelial cells, ductal smooth muscle cells in testis and epididymis. In the pubertal period, immunostaining of TLR2 and 7 started to be seen in primary spermatocytes, as well as other cells, in the testis. Narrow cells showed strong intracytoplasmic staining in the epididymis. In the postpubertal period, moderate to strong immunostaining of TLR2 and TLR7 was seen in spermatids at different developmental steps but weak immunoreaction in pachytene spermatocytes. Other cells in testis and epididymis showed variable immunostaining of TLR2 and 7. However, weak to moderate immunoreaction to TLR8 was detected in only interstitial cells in testis. In the mature period, immunostaining of TLR2, 7 and 8 tended to increase in different types of cells in testis and epididymis. Our findings suggest that expression of TLR2, 7 and 8 changed dynamically during postnatal development and increased towards mature period. We consider that TLR2, 7 and 8 might be associated with the regulation of spermatogenesis and the maintenance of innate immunity of testis and epididymis during postnatal development.

Keywords: *Toll-like receptors, Innate immunity, Testis, Epididymis, Postnatal development*

Postnatal Gelişim Döneminde Rat Testis ve Epididimisinde Toll-benzeri Reseptörler 2, 7 ve 8'in Ekspresyon Profilleri

Öz

Toll-benzeri reseptörler, çeşitli zararlı patojenlerin saldırısına yanıt olarak doğal bağışıklıkta önemli bir rol oynamaktadırlar. Postnatal gelişim boyunca rat testis ve epididimisinde TLR2, 7 ve 8 ifadesini araştırmayı amaçladık. Prepubertal dönemde TLR2 ve 7'nin, testis ve epididimisteki perităbüler miyoid hücreler, interstisyal hücreler, kan damarları, epitel hücreleri ve duktal düz kas hücrelerinde değişik şekillerde lokalize olduğu tespit edildi. Pubertal dönemde TLR2 ve 7 immunboyanması, testiste diğer hücrelerin yanı sıra primer spermatositlerde görülmeye başladı. Epididimiste dar hücreler, güçlü intrasitoplazmik boyanma gösterdi. Postpubertal dönemde farklı gelişimsel aşamalardaki spermatidlerde orta-güçlü derecede TLR2 ve 7 immünboyanması görülürken, pakiten spermatositlerde zayıf immünboyanma görüldü. Testis ve epididimisteki diğer hücre tipleri değişken derecelerde TLR2 ve 7 immünboyanması göstermiştir. Ancak testiste sadece interstisyal hücrelerde zayıf-orta derecede TLR8 immünreaksiyonu saptandı. Erişkin dönemde testis ve epididimisteki farklı hücre tiplerinde TLR2, 7 ve 8 immünboyanmasının artma eğiliminde olduğu dikkat çekti. Bulgularımız TLR2, 7 ve 8 ekspresyonunun postnatal gelişim sürecinde dinamik olarak değiştiğini ve erişkin döneme doğru arttığını göstermektedir. TLR2, 7 ve 8'in postnatal gelişim sürecinde spermatogenezin düzenlenmesi ve testis ile epididimisin doğal bağışıklığının sürdürülmesi ile ilişkili olabileceğini düşünüyoruz.

Anahtar sözcükler: *Toll-benzeri reseptörler, Doğal bağışıklık, Testis, Epididimis, Postnatal gelişim*

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INTRODUCTION

Pattern recognition receptors (PRRs) cover structurally divergent proteins, including Toll-like receptors (TLRs). PRRs recognize conserved moieties known as pathogen-associated molecular patterns (PAMPs) that are characteristic of specific pathogens. TLRs take an essential part in innate immunity [1]. TLRs mediate the induction of antimicrobial peptides in response to invasion of the bacterial, fungal and viral pathogens [2]. TLR family is composed of 13 members (TLR1-13) commonly found in many vertebrate species. TLR1-10 have been discovered in humans and TLR11-13 are confined to rodents [3]. Each TLR has been demonstrated to recognize specific components of pathogens. For instance, TLR2 recognizes microbial lipopeptides such as porin, peptidoglycan and lipoteichoic acid [4] while TLR7 and TLR8 recognize single-stranded RNA molecules (ssRNAs) [5-7]. Recognition of specific cell wall components with TLRs initiates a series of events containing various adaptor proteins and protein kinases, ultimately leading to the activation of immune response genes [8,9]. The targeted genes contain those encoding cytokines such as antimicrobial peptides, interleukin-12p40, interferon-beta, adhesion molecules, acute phase proteins, tumor necrosis factor-alpha (TNF-α), chemokines, and cyclooxygenase 2 [10]. The initial host defense against invading pathogens and opportunistic organisms culminates in the production of proinflammatory cytokines during an inflammatory response. They all together protect immediately hosts against pathogens and mount adaptive immune responses to those pathogens as well [8].

Microbial and pathogenic agents such as *Escherichia coli*, *Chlamydia trachomatis*, *Staphylococcus aureus* and *Neisseria gonorrhea* may infect the male reproductive tract and present a challenge for normal reproductive and endocrine functions. Epididymitis, which obstructs sperm movement and leads to inflammation, is due to the backward movement of microorganisms from the vas deferens [11]. Development of infection in the epididymis may form an epididymal abscess. Moreover, progressing infection may result in testicular involvement, eventually causing epididymo-orchitis or a testicular abscess [12,13]. As is the case with other systems, infections occurring in the male reproductive tract is anticipated mounting immune response through the activation of immune receptors, ultimately culminating in the induction of a lot of genes including the antimicrobial peptides and proteins [14]. For example, infection with *S. aureus* increased the expression of TLR2 in rat epididymis, suggesting that epididymal epithelium mounts an innate immune response through the activation of p38 MAPK and NF-κB after the increased expression of TLR2 [15].

Antimicrobial protection of immune-privileged organs such as testis and epididymis has become a recent active area of research. It has been reported that TLRs are expressed in different regions of the male reproductive tract [14,16,17]. Previous studies have focused mainly on mRNA expression of TLRs. However, cellular localization, distribution and expression patterns of TLRs such as TLR2, 7 and -8 have not been investigated in testis and epididymis of the rats at different periods of postnatal development. Therefore, we hypothesized that TLR2, 7 and 8 play essential roles in the pattern recognition during postnatal development of male reproductive tract. We here sought to identify the cell types expressing TLR2, 7 and 8 and to investigate their cellular localization, distribution and expression patterns in testis and epididymis of the rats by using immunohistochemistry.

MATERIAL and METHODS

Animal Materials and Experimental Design

Approval was obtained from Ankara University Local Ethics Committee for Animal Experiments (approval number #2014-18-128). A total of 24 of Wistar albino rats (200-300 g) at different stages of postnatal development were used in this study. Rats were divided into four groups, each of which composes of six rats. Groups were designated as prepubertal (postnatal 5 days, PND5), pubertal (PND20), postpubertal (PND50) and mature (PND70) periods. The animals were maintained at room temperature (20-24°C) in a 12-h light/dark circle and fed a standard diet and water *ad libitum*. By the end of each developmental period, all the rats were euthanized under anesthesia and testicular and epididymal tissues samples were harvested. These processes were done at the same time interval to prevent any alteration resulted from biological rhythm of animals.

Tissue Processing

Tissue processing was made as previously described [18]. Shortly, the testicular and epididymal samples were fixed in Bouin's solution, passed through a graded series of alcohols, methyl benzoate and benzene, and embedded in paraplast.

Immunohistochemistry

Immunohistochemistry was performed employing the streptavidin-biotin peroxidase technique [19]. A 5-µm thick sections were affixed onto Poly-Lysine-coated slides (Thermo Fisher Scientific). The sections were kept in 3% hydrogen peroxide in methanol/PBS for 20-30 min to quench endogenous peroxidase activity. Antigen retrieval was performed with citrate buffer (#AP-9003-500, Thermo Fisher Scientific Lab Vision) to unmask antigenic epitopes. They were then incubated with primary antibodies (TLR2, 1/300, #bs-1019R; TLR7, 1/300, #ab45371; TLR8, 1/300, PA5-20056) at +4°C for 16 h. Following the routine procedure, they were treated with AEC chromogen (#TA-060-HA). The sections were counterstained with Gill's II hematoxylin and coverslipped with water-based mounting medium (#TA-125-UG). Results of immunostaining were analyzed by two blind observers under a light microscope (DM 2500, Leica, Germany) with a digital camera (DFC450, Leica, Germany). Images were captured using Leica Application Suite software.

RESULTS

No staining was observed in negative control sections when normal rabbit serum (#sc-2027) was used instead of the primary antibodies. Representative sections for immunolocalization of TLR2, TLR7 and TLR8 are presented in the testis and epididymis of rats *(Fig. 1, 2, 3, 4, 5, 6)*. Expression pattern of TLRs, especially of TLR2 and TLR7, tended to increase from prepubertal period to mature period. Expression of TLR8 was not detected in testis and epididymis at the prepubertal, pubertal and postpubertal periods, except for interstitial cells at postpubertal period. In general, staining intensity of TLR2 and TLR7 were much more pronounced than that of TLR8 at all the periods.

TLR2 Immunohistochemistry

PND 5: Peritubular myoid cells, some interstitial cells and blood vessels showed weak, moderate and strong immunoreactivity, respectively. However, any immune reaction was not detected in immature Sertoli cells and gonocytes *(Fig. 1-a)*. In epididymis, epithelial cells, ductal smooth muscle

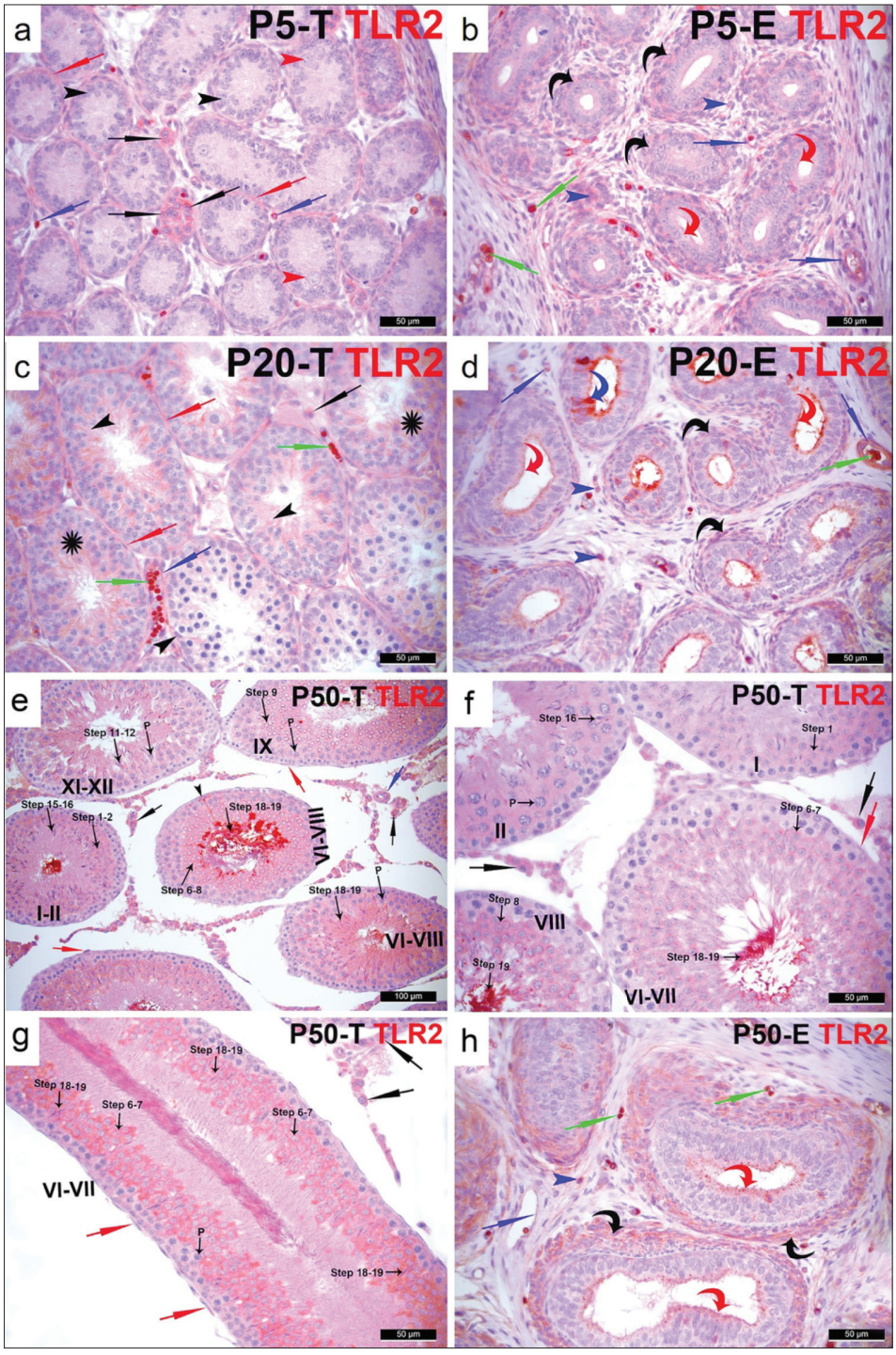

Fig 1. TLR2 immunostaining in the rat testis and epididymis at PND5, 20 and 50. **(a, c)** In the testis, no staining in gonocytes *(red arrowheads)* and Sertoli cells *(black arrowheads)*. Positive staining in primary spermatocytes *(asterisks)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)* and blood vessels *(blue arrows)*. **(b, d)** In the epididymis, positive staining in epithelial cells *(red curved arrows)*, ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)*, vessel walls *(blue arrows)* and blood cells *(green arrows)*. **(e-g)** Positive staining in pachytene (P) spermatocytes *(black arrows)*, spermatids at different steps *(black arrows)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)*. **(h)** In the epididymis, positive staining in epithelial cells (red curved arrows), ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)*, vessel walls *(blue arrows)* and blood cells *(green arrows)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale bars: 100 µm (E), 50 µm (A, B, C, D, F, G, H)

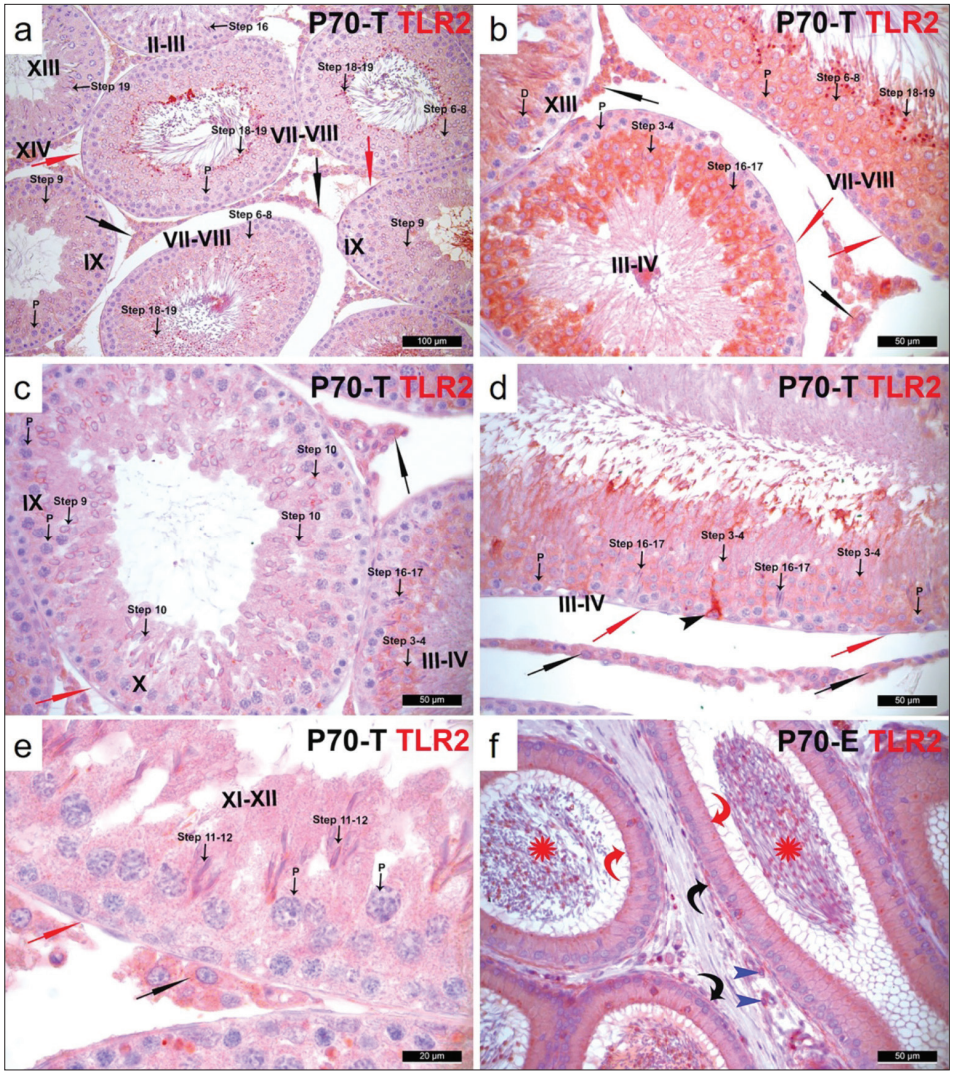

Fig 2. TLR2 immunostaining in the rat testis and epididymis at PND70. **(a-e)** Positive staining in pachytene (P) spermatocytes *(black arrows)*, spermatids at different steps *(black arrows)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)*. **(d)** In the epididymis, positive staining in epithelial cells (red curved arrows), ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)* and spermatozoa *(red asterisks)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale bars: 100 µm (A), 50 µm (B, C, D and F), 20 µm (E)

cells and some interstitial cells had weak immunostaining while vessel walls and some blood cells showed strong staining *(Fig. 1-b)*.

PND 20: In testis, we observed weak staining in primary spermatocytes and some interstitial cells, weak to moderate staining in peritubular myoid cells and apical surface of Sertoli cells, moderate staining on vessel wall and strong staining on some blood cells *(Fig. 1-c)*. In epididymis, apical surface of all epithelial cells had moderate to strong staining while some narrow cells exhibited strong intracytoplasmic staining. Vessel wall and some interstitial cells had moderate immunostaining. Some blood cells showed strong immunoreactivity. Weak staining was detected in ductal smooth muscle cells *(Fig. 1-d)*.

PND 50: We found moderate to strong immunoreaction in elongated spermatids and weak to moderate immunoreaction in round spermatids at different spermatogenic stages. Pachytene spermatocytes exhibited weak immunoreaction. Peritubular myoid cells had weak to moderate immunoreaction. Moderate immunoreaction was detected in some interstitial cells and apical surface of Sertoli cells *(Fig. 1-e,f,g)*. In epididymis, moderate immunoreaction was seen in ductal smooth muscle cells and some interstitial cells and on apical surfaces of epithelial cells. Some blood cells showed strong immunoreaction *(Fig. 1-h)*.

PND 70: Compared to earlier periods, immunostaining tended to increase in testis and epididymis. We observed moderate to strong immunoreaction in elongated and round spermatids at different developmental steps while pachytene spermatocytes exhibited weak to moderate immunoreaction. We found weak immunoreaction in peritubular myoid cells but moderate immunoreaction in interstitial cells *(Fig. 2-a,b,c,d,e)*. In epididymis, moderate to strong immunoreaction was seen in ductal smooth muscle cells, luminal spermatozoa, some interstitial cells and the apical surfaces of epithelial cells *(Fig. 2-f)*.

TLR7 Immunohistochemistry

PND 5: We observed moderate immunostaining in immature Sertoli cells and peritubular myoid cells although no reaction was present in gonocytes. Vessel wall and some interstitial cells showed weak to moderate immunoreaction *(Fig. 3-a)*.

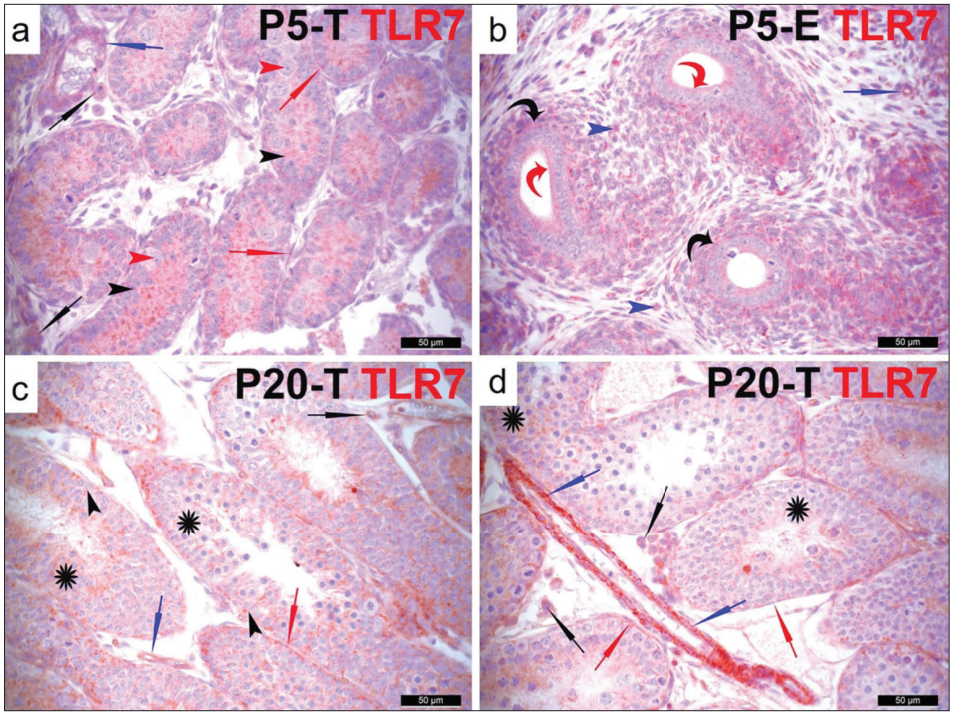

Fig 3. TLR7 immunostaining in the rat testis and epididymis at PND5 and 20. **(a, c, d)** In the testis, no immunoreaction in gonocytes *(red arrowheads)* and Sertoli cells *(black arrowheads)*. Positive staining in primary spermatocytes *(asterisks)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)* and blood vessels *(blue arrows)*. **(b)** In the epididymis, positive staining in epithelial cells *(red curved arrows)*, ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)* and vessel walls *(blue arrows)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 50 µm

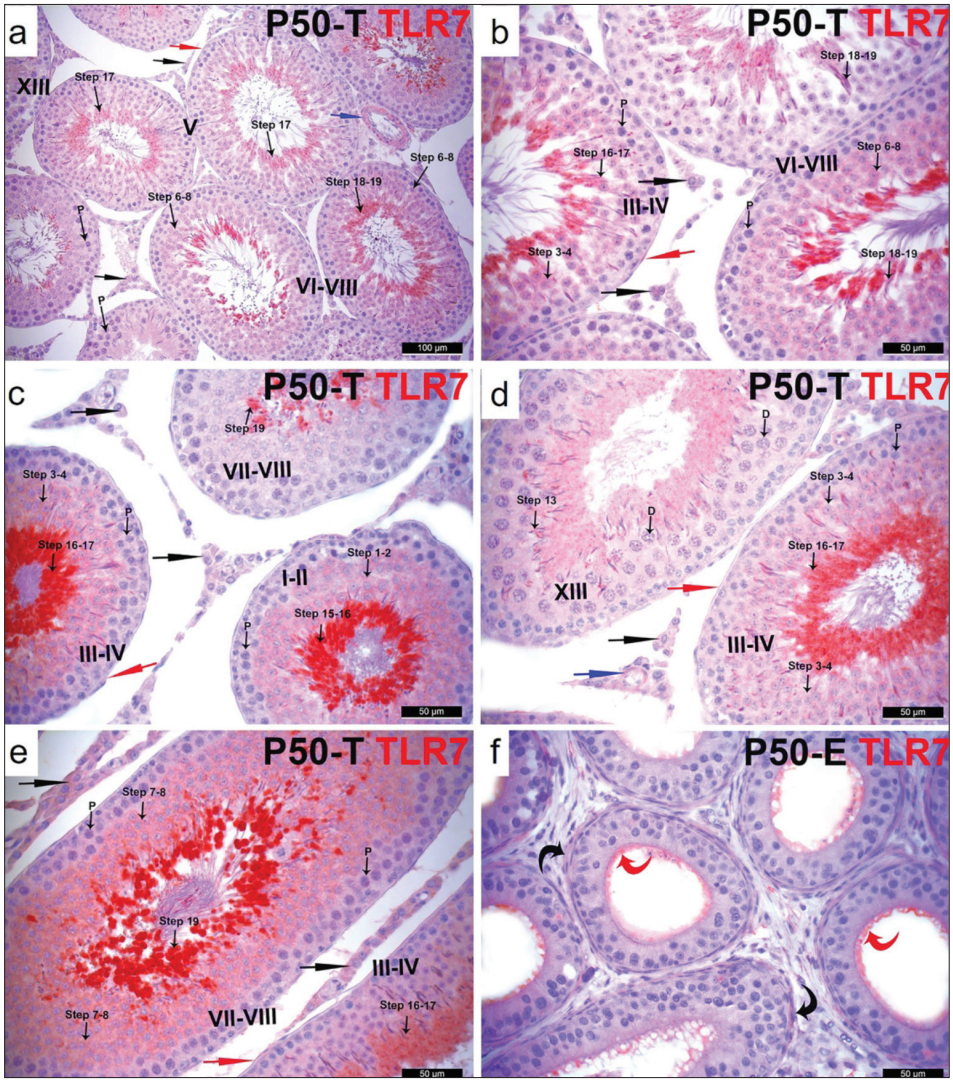

Fig 4. TLR7 immunostaining in the rat testis and epididymis at PND50. **(a-e)** Positive staining in pachytene (P) spermatocytes *(black arrows)*, spermatids at different steps *(black arrows)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)*. **(f)** In the epididymis, positive staining in epithelial cells *(red curved arrows)* and ductal smooth muscle cells *(black curved arrows)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 100 µm (A), 50µm (B, C, D, E and F)

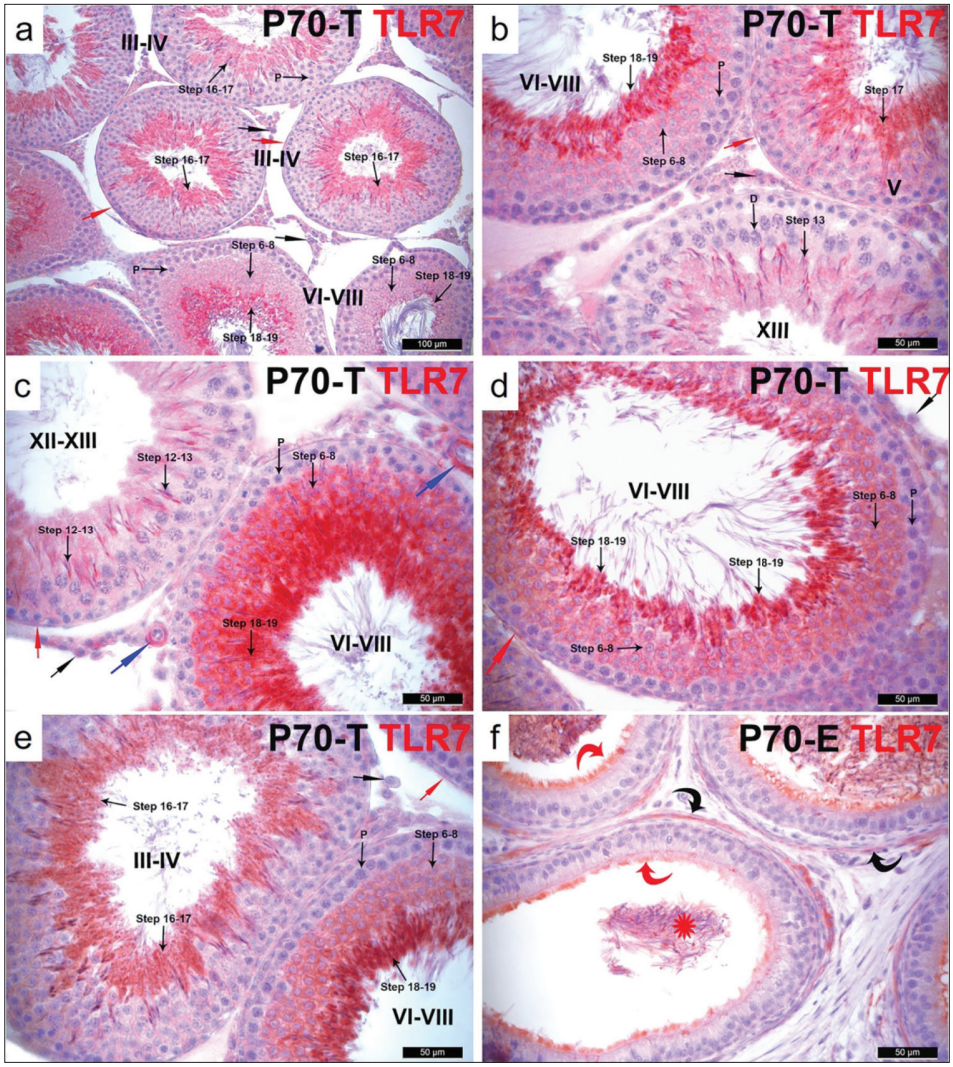

Fig 5. TLR7 immunostaining in the rat testis and epididymis at PND70. **(a-e)** Positive staining in pachytene (P) spermatocytes *(black arrows),* spermatids at different steps *(black arrows),* peritubular myoid cells *(red arrows),* interstitial cells *(black arrows).* **(d)** In the epididymis, positive staining in epithelial cells *(red curved arrows),* ductal smooth muscle cells *(black curved arrows)* and spermatozoa *(red asterisks).* Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 100 µm (A), 50 µm (B, C, D, E, F)

In epididymis, weak to moderate immunoreaction was observed in interstitial cells and vessel wall while ductal smooth muscle cells and apical surface of all epithelial cells exhibited moderate immunostaining *(Fig. 3-b)*.

PND 20: We found moderate immunostaining in Sertoli cells, primary spermatocytes, some interstitial cells, peritubular myoepithelial cells, and strong staining in vessel wall *(Fig. 3-c,d)*. In epididymis, immunoreaction was similar to that of prepubertal period.

PND 50: We detected strong immunoreaction in elongated spermatids and moderate immunoreaction in round spermatids at different developmental steps. Pachytene spermatocytes, peritubular myoid cells and interstitial cells showed weak immunoreaction. We found moderate to strong immunoreaction in the apical surface of Sertoli cells but moderate immunostaining in vessel wall *(Fig. 4-a,b,c,d,e)*. In epididymis, weak immunoreaction was seen in ductal smooth muscle cells while moderate staining was present on the apical surfaces of epithelial cells and vascular smooth muscle cells. On the other hand, interstitial cells stained heterogeneously weak *(Fig. 4-f)*.

PND 70: Compared to earlier periods, we detected the increased TLR7 expression in testis and epididymis. We observed strong immunoreaction in elongated spermatids and moderate to strong immunoreaction in round spermatids at different developmental steps. Pachytene spermatocytes and peritubular myoid cells showed weak immunoreaction. Vessel wall had strong immunostaining. Interstitial cells exhibited weak to moderate immunoreaction *(Fig. 5-a,b,c,d,e)*. In epididymis, moderate immunoreaction was observed in ductal smooth muscle cells, luminal spermatozoa and the apical surfaces of epithelial cells whereas interstitial cells were weakly stained *(Fig. 5-f)*.

TLR8 Immunohistochemistry

PND 5 and *20:* We did not detect any positive immunoreaction in the testis and epididymis *(Fig. 6-a,b,c,d)*.

PND 50: We detected weak to moderate immunoreaction in only interstitial cells in testis but weak staining in epididymis *(Fig. 6-e,f)*.

PND 70: We found weak immunolabelling in elongated

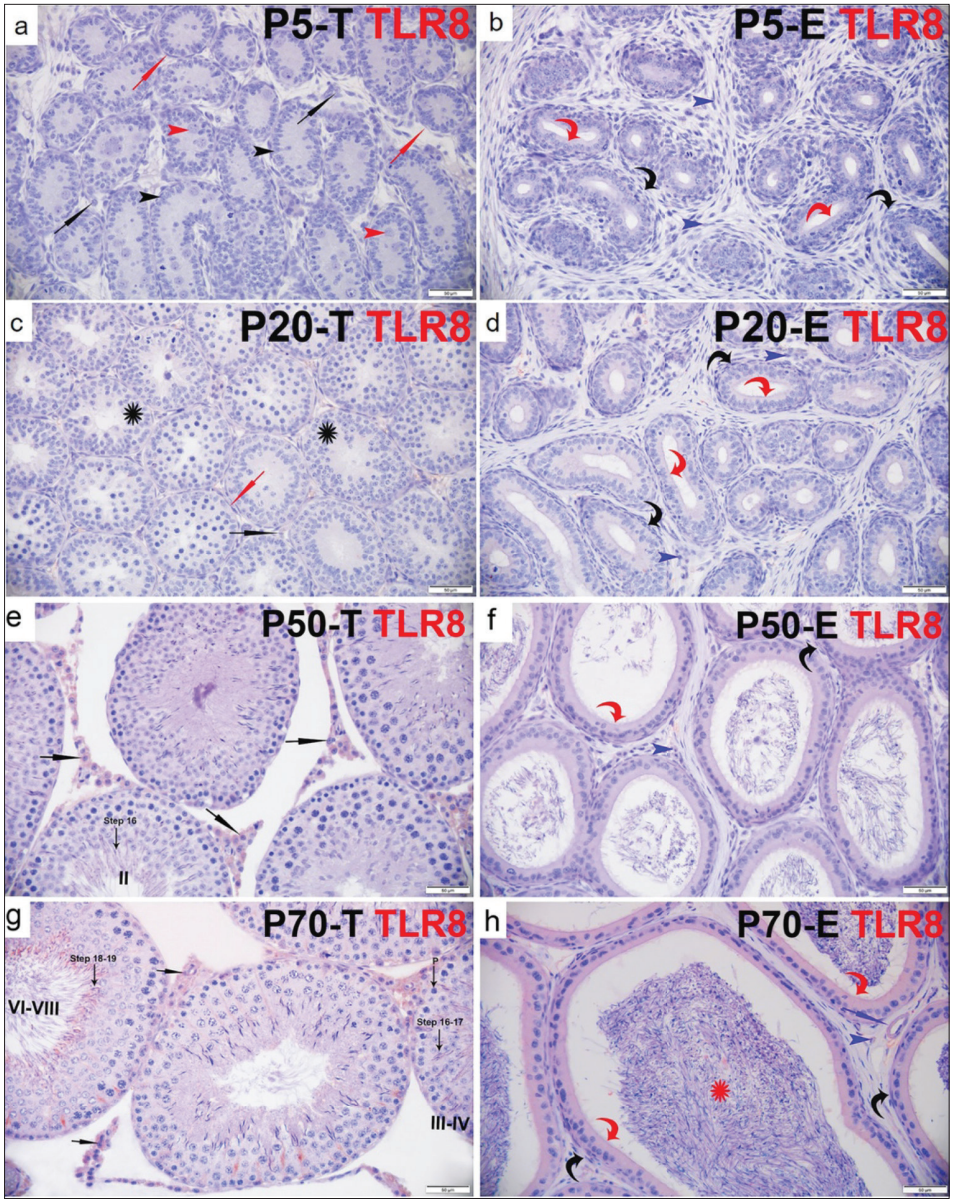

Fig 6. TLR8 immunostaining in the rat testis and epididymis at PND5, 20, 50 and 70. **(a-c)** No staining in gonocytes *(red arrowheads),* Sertoli cells *(black arrowheads),* primary spermatocytes *(asterisks),* peritubular myoid cells *(red arrows)* and interstitial cells *(black arrows).* **(b-d)** In the epididymis, no staining in epithelial cells *(red curved arrows),* ductal smooth muscle cells *(black curved arrows)* and interstitial cells *(blue arrowheads).* (e-g) In testis, positive staining in interstitial cells *(black arrows),* Sertoli cells *(black arrowhead)* and spermatids at different steps *(black arrows).* **(f-h)** In epididymis, positive staining in epithelial cells *(red curved arrows),* ductal smooth muscle cells *(black curved arrows),* interstitial cells *(blue arrowhead),* vessel wall *(blue arrow)* and spermatozoa *(red asterisk).* Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 50 µm

spermatids and interstitial cells but weak to moderate immunolabelling in Sertoli cells. In epididymis, there was weak immunoreaction in epithelial cells, ductal smooth muscle cells and vessel wall *(Fig. 6-g,h).*

DISCUSSION

We here investigated the expression pattern of TLR2, 7 and 8 proteins in rat testis and epididymis during postnatal development. The testicular and epididymal expression of TLRs, notably TLR2 and TLR7, significantly differed according to the developmental periods.

Many molecules such as androgens are required for postnatal differentiation and maintenance of testicular and epididymal structure [20]. In addition, TLRs involving in innate immunity may play key roles during postnatal differentiation. A study has performed a comprehensive analysis of TLR expression and distribution in the rat testis and epididymis [21]. They reported that TLR2 was abundantly expressed in testis and epididymis, TLR7 was mainly present in the testis despite of being weakly expressed in the epididymis, and TLR8 exhibited weak expression in testis and epididymis, which are consistent with our results. In addition, it has been showed that TLR2 mRNA is more produced by human testis than TLR7 mRNA and TLR8 mRNA, with more expression of TLR7 mRNA than that of TLR8 mRNA [22], which are also largely in concordance with the expression of TLR2, TLR7 and TLR8 proteins in our study. Unlike previous researches, it has been reported that testicular expression of TLR2 decreased towards further developmental periods [14]. This difference may arise from a higher rate of TLR2 mRNA destabilization or degradation than those of its protein, presumably through mechanisms that are dictated by miRNAs or from posttranscriptional and/or posttranslational modifications [23,24].

Previous studies reported that the existence of other innate molecules inc luding MYD88 [21] and CD14 [25] in the male tract along with the TLRs [16] gives an indication that an invasion of microbial or pathogenic agents could lead to the activation of immune effector pathways that have the capacity to induce the production and secretion of antimicrobial peptides and cytokines. In the present study, the increased expression of TLR2, TLR7, and TLR8 in the transition from the prepubertal period to mature period might show that the male reproductive tract is ready to respond to any infection that could be developed.

It has been found that, in human testis, TLR2 was expressed in peritubular cells, Sertoli cells and interstitial cells [26] in accordance with our data. In addition, a recent study has shown variable immunostaining for TLR2 in the epididymal epithelial cells, interstitial cells, ductal smooth muscle cells and sperm cells of adult tom cats, with marked staining on the apical surfaces of epithelial cells [17]. They did not observe any difference between epididymal regions. Their findings are consistent with our results of testis and epididymis. However, we observed different from their study that epididymal expression of TLR2 tended to increase towards mature period. Yet another study reported that mouse Sertoli cells express TRL2 at high levels and TLR7 at lower levels, but they do not express TLR8 [27]. Their results are in line with our findings of TRL2 and TRL7 at PND5 and 20 in terms of animal ages since they used Sertoli cells from 3-week old mice. However, we observed TLR8 expression in Sertoli cells at PND70. We speculated that increased TLR expression in Sertoli cells may serve a protective purpose during further developmental periods, suggesting that Sertoli cells provide the defense machinery for developing germ cells against auto- and alloantigens.

There is limited research on the expression of TLR2, 7 and 8 in male germ cells. We observed that expression of TLR2, 7 and 8 showed a tendency to increase after the transition from gonocytes and spermatogonia to spermatocytes and spermatids and their expression was stronger in spermatids than other spermatogenic cell types. This may mean that their expression initiated in response to the formation of functional spermatogenic cells. Their enhanced expression towards the mature period could also provide a protective mechanism for spermatozoa capable of fertilizing ovum against pathogenic microbial agents, and protect the female genital tract against these agents.

In conclusion, our results show that expression of TLR2, 7 and 8 changed dynamically during postnatal development and increased towards the mature period. We consider that TLR2, 7 and 8 might be associated with the regulation of spermatogenesis and the maintenance of innate immunity of testis and epididymis during postnatal development. However, further studies are required to better understand their roles during the postnatal development of testis and epididymis.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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Research Article

Antimicrobial Effects of *Thymus vulgaris, Cinnamomum zeylanicum* and *Zingiber officinale* Essential Oils on *Salmonella enterica* serovar Enteritidis Infections

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Abstract

Salmonella enterica serovar Enteritidis infections are among the leading causes of human foodborne illness mainly due to the consumption of contaminated poultry meat and eggs. Therefore, the aim of this study was to investigate the antimicrobial effects of essential oils (EOs) derived from *Thymus vulgaris* (thyme), *Cinnamomum zeylanicum* (cinnamon) and *Zingiber officinale* (ginger) on these infections. These EOs were added to *Salmonella enterica* ser. Enteritidis cultures in 96-well microplates in a sealed pouch and incubated at 37°C for 24 h minimum inhibitory concentration was measured to determine their antimicrobial effects. Ciprofloxacin (1 mg/mL) added to the culture medium served as the positive control to test the antibacterial effect. In this in vitro cell culture study, the cytotoxic effects of all EOs on healthy fibroblasts were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide cell viability test. Cinnamon EO showed antibacterial effects at all concentrations, whereas ginger EO was effective only in combination with thyme EO at 50% concentration. Further, thyme EO was found to be bactericidal at 50% concentration and bacteriostatic at 25% concentration. None of the EOs were cytotoxic to fibroblasts. In conclusion, cinnamon EO is highly effective and safe for the treatment of *Salmonella enterica* ser. Enteritidis infections, followed by thyme EO.

Keywords: *Antibacterial effects, Thymus vulgaris, Thyme, Cinnamomum zeylanicum, Cinnamon, Zingiber officinale, Ginger, Essential oil, Salmonella enterica*

Thymus vulgaris, Cinnamomum zeylanicum ve *Zingiber officinale* Uçucu Yağlarının *Salmonella enterica* Serovar Enteritidis Üzerindeki Antimikrobiyal Etkileri

Öz

Salmonella enterica serovar Enteritidis enfeksiyonları, esas olarak kontamine kümes hayvanı eti ve yumurta tüketimi nedeniyle insan gıda kaynaklı hastalıkların önde gelen nedenleri arasındadır. Bu nedenle, bu çalışmanın amacı *Thymus vulgaris* (kekik), *Cinnamomum zeylanicum* (tarçın) ve *Zingiber officinale*'den (zencefil) elde edilen esansiyel uçucu yağların (EY) bu enfeksiyonlar üzerindeki antimikrobiyal etkilerini araştırmaktır. Bu EY'lar *S. enterica* serovarının bulunduğu 96 oyuklu mikroplakalarda enteritidis kültürlerinin bulunduğu ortamda 37°C'de 24 saat inkübe edildi. Antimikrobiyal etkileri belirlemek için minimal inhibitör konsantrasyon ölçüldü. Kültür ortamına eklenen siprofloksasin (1 mg/mL), antibakteriyel etkiyi test etmek için pozitif kontrol görevi gördü. Bu *in vitro* hücre kültürü çalışmasında, tüm EY'ların sağlıklı fibroblastlar üzerindeki sitotoksik etkileri 3-(4,5-dimetiltiyazol-2-il) -2,5-difeniltetrazolium-bromür hücre canlılığı testi kullanılarak araştırıldı. Tarçın EY'ı tüm konsantrasyonlarda antibakteriyel etkiler gösterirken, zencefil EY'ı sadece %50 konsantrasyonda kekik EY ile kombinasyon halinde etkiliydi. Ayrıca kekik EY'nın %50 konsantrasyonda bakterisidal ve %25 konsantrasyonda bakteriyostatik olduğu bulundu. Hiçbir EY fibroblast hücrelerine sitotoksik değildi. Çalışmamızın bulgularında tarçın EY'nın *S. enterica* serovarı tedavisinde oldukça etkili ve güvenli olduğunu göstermekte olup bunu kekik EY'ı takip etmektedir. Hiçbir EY fibroblast için sitotoksik değildi. Sonuç olarak, tarçın EY'ı *Salmonella enterica* ser. Enteritidis tedavisi için oldukça etkili ve güvenlidir. Enteritidis enfeksiyonlarına etkinlikte bunun ardından kekik EY'ı gelmektedir.

Anahtar sözcükler: *Antimikrobiyal etki, Thymus vulgaris, Kekik, Cinnamomum zeylanicum, Tarçın, Zingiber officinale, Zencefil, Esansiyel yağ, Salmonella enterica*

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INTRODUCTION

Salmonellosis caused by *Salmonella* spp. is a widely reported zoonotic disease, and it is transmitted to humans through the consumption of raw animal food products including poultry meat and eggs. *Salmonella* infections are highly prevalent in humans, animals and birds. Therefore, these infections are a major concern for public health, animals and the food industry worldwide [1]. *Salmonella enterica* serovar Enteritidis has been associated with a number of outbreaks of salmonellosis in humans.

In recent years, natural antimicrobials such as plant essential oils (EOs) and extracts have been considered popular alternatives to commercially used chemicals such as chlorine and hydrogen peroxide, which have harmful effects [2,3]. Plant EOs and extracts have been used in food preservation, pharmaceuticals, alternative medicine and natural therapies. Božik et al.[4] reported that there are potentially six plant EOs-antimicrobial volatile substances derived from plants with beneficial effects-which are suitable alternatives to synthetic pesticides and food preservatives, which can be used for the treatment of microbial infections [4]. These EOs are derived from cinnamon *(Cinnamomum zeylanicum* Nees.), thyme *(Thymus vulgaris* L.), oregano *(Origanum vulgare* L.), clove *(Syzygium aromaticum* L.), lemongrass [*Cymbopogon citratus* (DC) Stapf.] and ginger *(Zingiber officinale* Rosc.) [4]. For the study of the effects of the study EOs on *S. enteritidis*, the three plants were selected because scientific studies have commenced on this issue recently [5,6], although they are very rare.

The aim of this study was to investigate the effect of thyme, cinnamon and ginger EOs on *S. enterica* ser. Enteritidis cultures and to elucidate their cytotoxic effects on healthy fibroblasts. Elucidation of the antimicrobial effects of these EOs can facilitate the design of suitable therapies for the treatment of *S. enterica* ser. Enteritidis infections. Through this study, we hope to contribute to the literature in this field.

MATERIAL and METHODS

Plant Materials

The plant materials used in this study included *T. vulgaris* (thyme), *C. zeylanicum* (cinnamon) and *Z. officinale* (ginger). This study was conducted at the Kırıkkale University Scientific and Technological Research Application and Research Center.

EO Extraction: This *in vitro* study was approved by the Ministry of Agriculture, and three readymade test EO extracts from the same commercial centre, which are commercially available and sold under a specific barcode number, were used. Therefore, any powdered samples could not be extracted with methanol. Density (d) of EOs calculating with Mettler Toledo density meter was as follows: *C. zeylanicum* = 0.889 g/mL, *T. vulgaris* = 0.912 g/mL and *Z. officinale* = 0.868 g/mL.

Test Organisms: The *Salmonella enteritidis* bacterial strain used in this study was obtained from the Kırıkkale University Scientific and Technological Research Application and Research Center.

Cell Line

L929 (a healthy mouse adipose fibroblast cell line) cells that were frozen at -80°C in cryotubes in the cell library of the Kırıkkale University Scientific and Technological Research Application and Research Centre Biocompatibility Laboratory were used.

Minimum Inhibitory Concentration (Antimicrobial Effect)

In 96-well microplates, 90 µL of Mueller–Hinton broth medium was first introduced into all wells. Subsequently, 90 µL of the three EOs was added (cinnamon, ginger, thyme and their mixtures) to the first well in each of the 6 rows to achieve 1:1 concentration. Thereafter, EOs were added at different concentrations in the following ratios: 1:2 (50%), 1:4 (25%), 1:8 (12.5%), 1:16 (6.25%), 1:32 (3.13%), 1:64 (1.56%) and 1:128 (0.78%).

The concentration of 24-h fresh *Salmonella enterica* ser. Enteritidis culture was adjusted to 0.5 McFarland turbidity standard and diluted to a ratio of 1:100. Approximately 10 µL of this inoculum was added to each well to ensure that the final concentration was 10^5 microorganisms/well. For measuring the antibacterial effect, ciprofloxacin (1 mg/mL) was used as a positive control in a well containing only the medium. Thereafter, the 96-well microplates were placed in a sealed pouch and incubated (Binder CB150, USA) at 37°C for 24 h to observe the antimicrobial effects of the EOs.

Cytotoxicity Analysis

For cytotoxicity measurements, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) cytotoxicity test was performed. MTT was performed according to the ISO 10993-5:2009 standards. For adjusting cell density, 100 µL of Dulbecco's modified Eagle's medium (DMEM) was added to 10^5 L929 cells/well in a 96-well microtiter culture plate. L929 cells were incubated for 24 h at 37°C in 5% CO_2. Thereafter, EOs were added at the following different concentrations: 1:1 (100%), 1:2 (50%), 1:4 (25%), 1:8 (12.5%), 1:16 (6.25%), 1:32 (3.13%), 1:64 (1.56%) and 1:128 (0.78%).

Negative wells (containing cells only), positive wells (containing 20% DMSO plus EO with L929 cells), control wells and blind wells (DMEM only) were used in triplicates. Cells were incubated for 24 h at 37°C in 5% CO_2. At the end of the 24-h incubation, the medium was carefully removed from the 96-well microtiter culture plate and 10

µL of MTT solution was added to each well and incubated at 37°C for 1-4 h. After incubation, the incubation buffer was removed from the wells, cells were shaken by adding 100 µL of isopropanol to each well, and absorbance was measured on a microplate reader (Biotek PowerWave XS2) at a wavelength of 570 nm decrease in viability compared with that of cells grown in the blind wells is expressed as percentage. The result of the visually control and with sowing methods obtained four different results.

RESULTS

Antimicrobial Effect of the Three EOs and Their Mixtures

Cinnamon EO showed an antimicrobial effect on *S. enterica* ser. Enteritidis infections at all tested concentrations. Although thyme EO had bactericidal activity against *S. enterica* ser. Enteritidis infections at 50% concentration, it showed bacteriostatic activity at 25% concentration. Ginger EO did not show any antimicrobial effect. Further, cinnamon-thyme and cinnamon-ginger EO mixtures were found to have antimicrobial effects at all concentrations. However, the antimicrobial effect of thyme-ginger EO mixture was only found at 50% concentration. Regarding the *S. enteritidis* strain, antimicrobial effect and minimum bactericidal concentration *(Fig. 1)* of cinnamon EO (50%-0.78%), as well as its antimicrobial effect and MIC value (0.39%-0.006%, *Fig. 2)*, were detected.

Cytotoxic Effect of EOs

Fig. 3 shows the cytotoxic activity of all EOs. Our study showed that these EOs were not cytotoxic to fibroblasts at any concentration. No significant findings were obtained in cytotoxicity analysis.

DISCUSSION

Salmonellosis is a major zoonotic disease [7] which is prevalent worldwide. Marus et al.[7] analysed the data reported by the Centres for Disease Control and Prevention through the National Outbreak Reporting System from 2009 to 2014, with the primary mode of transmission listed as 'animal contact' or 'food contaminated'. They found 484 outbreaks through animal contact or foodborne transmission, of which 99 (20.5%) resulted from *Salmonella* transmission through animal contact and 385 (79.5%)

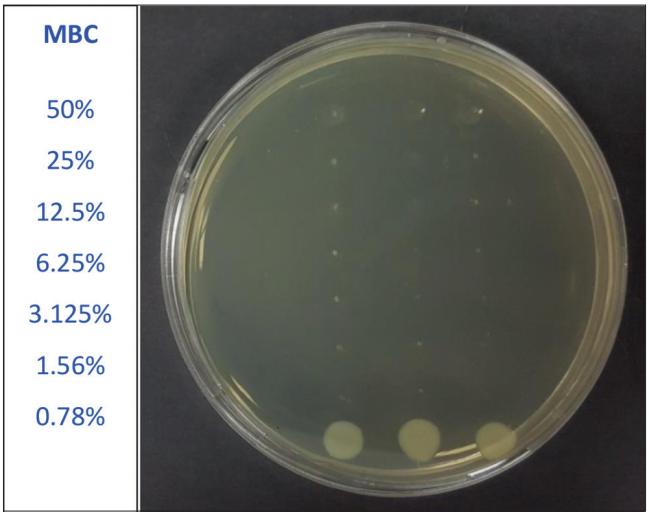
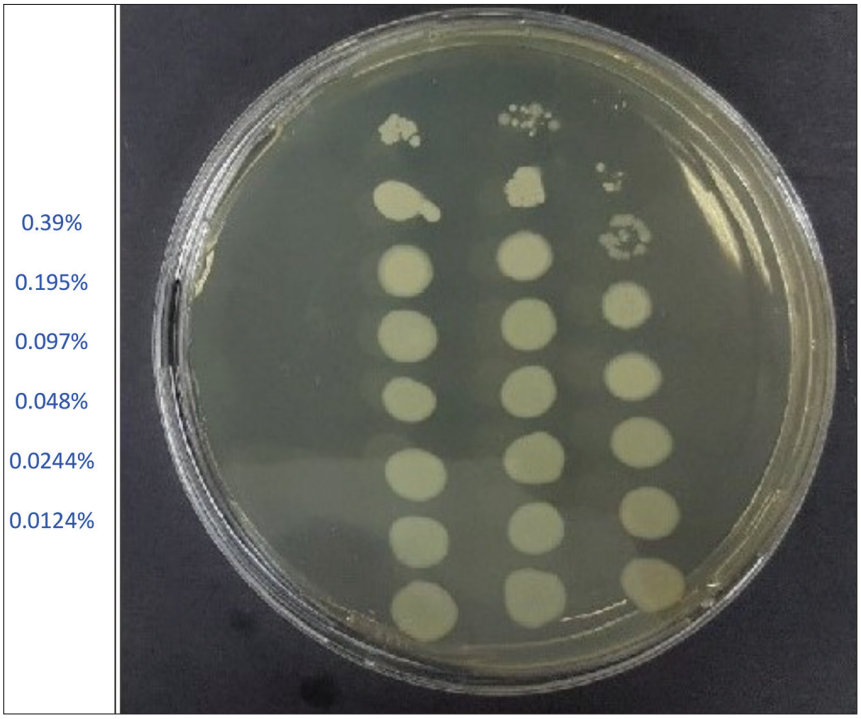


Fig 1. Antimicrobial effectand minimum bactericidal concentrationof cinnamon essential oil (50%-0.78%) inthe studied*Salmonella enteritidis* strain. Minimum inhibitory concentration: 0.39%-0.006%.

Fig 2. Antimicrobial effectand minimum inhibitory concentration of cinnamon essential oil (0.39%-0.006%) inthe studied *Salmonella enteritidis* strain



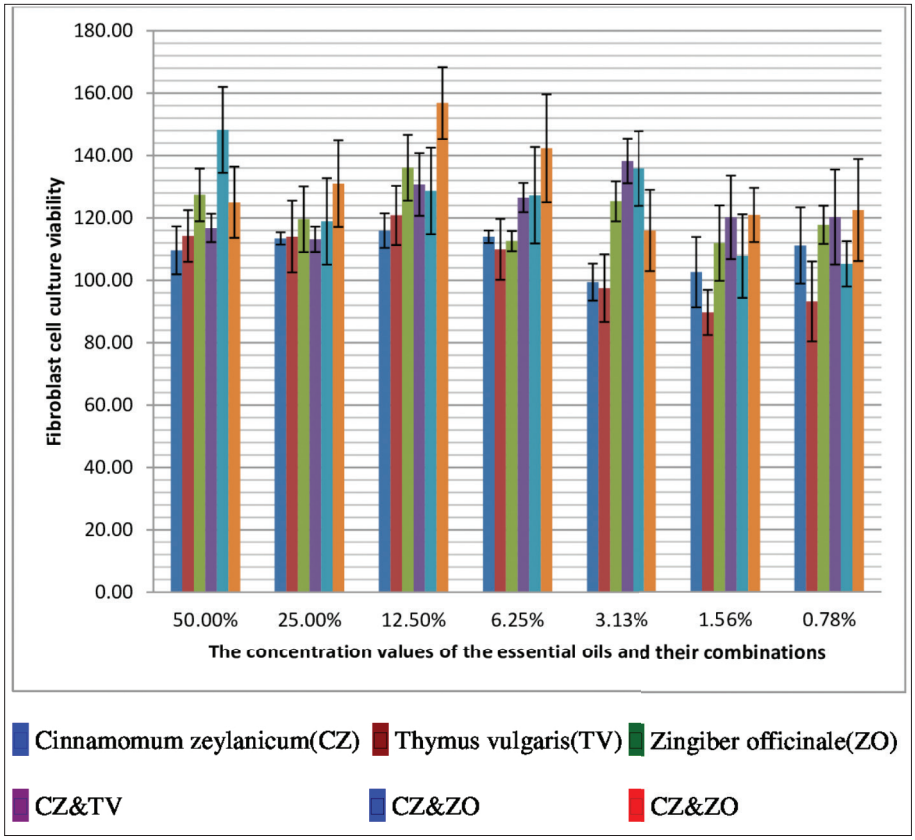


Fig 3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromidecell viability test results for cytotoxicity. The cytotoxic actiity of all studied EOs. Our study showed that these EOs were not cytotoxic to fibroblasts at any concentration. No significant findings were obtained in cytotoxicity analysis.

from the foodborne transmission; these resulted in 3.604 (19.8%) and 13.568 (80.2%) illnesses, respectively [7,8]. Giacometti et al.[9] reported highly suspected cases of salmonellosis in two cats fed a commercial raw meat-based diet [9]. Further, Tomaštíková et al.[10] reported a case wherein salmonellosis was transmitted from a reptile to an infant through indirect contact. Thus, salmonellosis can be transmitted through direct or indirect contact or through the consumption of contaminated poultry meat or eggs.

Some plant EOs have been reported to exhibit antimicrobial properties, and they are potential sources of novel antimicrobial compounds, particularly against bacterial pathogens. Warnke et al.[11] showed that plant EOs are a cheap and effective antiseptic treatment option, even for antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* and *S. enterica*. Zhu et al.[12] showed that carvacrol and cinnamaldehyde incorporated into apple-, carrot- and hibiscus-based edible films could inactivate *Salmonella* Newport in bagged organic leafy greens [12]. Al-Bayati [13] showed that EOs and methanol extracts derived from the aerial parts of *T. vulgaris* and *Pimpinella anisum* seeds had high inhibitory effects on most pathogenic bacteria such as *Bacillus cereus*, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Typhimurium and *Pseudomonas aeruginosa*, which are resistant to the standard antibiotic Maxipime [13]. Renata et al.[14] investigated the growth and survival of *Escherichia coli* 0157 and *S. enterica* ser. Enteritidis in the presence of garlic, ginger, mustard and cloves. Experiments performed in broth model systems supported with 0.25%-1% garlic and cloves showed bacteriostatic and bactericidal activities against both microorganisms.

Clove was the most effective antimicrobial agent, followed by garlic. However, mustard and ginger showed only little bacteriostatic activity [14]. Rosti and Gastaldi [15] reported a case in 2005 of the therapeutic effect of *C. zeylanicum* on an infant. The infant's mother had eaten raw seafood, and this exclusively breastfed 4-month-old infant became a carrier of *S. enterica* ser. Enteritidis. Stool samples of both mother and infant were positive for *S. enterica* ser. Enteritidis. However, they reported that chronic *Salmonella* carriage in the infant disappeared after cinnamon administration. Boskovic et al.[16] estimated the antibacterial effect of thyme EO (TEO) on four *Salmonella* serovars *(Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella montevideo* and *Salmonella infantis)* which were experimentally inoculated (10^6 CFU/g). The highest antibacterial effect was achieved by the combination of MAP and 0.9% TEO. They stated that all TEO concentrations in the studied pork had significant antibacterial effects ($P<0.05$). Travis et al.[17] reported that food poisoning outbreaks resulting from *Salmonella* growing on vegetables emphasise the need for the knowledge of pathogen evolution and adaptation in developing appropriate countermeasures for the prevention and policy development [17]. Therefore, the findings of this study are important in terms of both public health and prevention of zoonotic diseases.

Our study showed that cinnamon and thyme EOs had an effective antibacterial effect on *S. enterica* ser. Enteritidis. Ginger and thyme EOs were effective against *S. enterica* ser. Enteritidis at 50% concentration, but ginger EO alone did not have any antimicrobial effect, as shown in previous studies [11,13]. Pure *C. zeylanicum* and pure *T. vulgaris* or their mixtures were found to be effective against *S. enterica* ser. Enteritidis at ≤50% concentration, and these EOs and their mixtures can be considered in the development of therapeutic strategies for salmonellosis because they were not found to be cytotoxic to the fibroblast cell line.

Our *in vitro* study results confirm that the main EO which is effective and safe for the treatment of salmonellosis caused by *S. enterica* ser. Enteritidis is cinnamon EO, followed by TEO. It would be better if the extraction was performed in our laboratory, which is a limitation of this study. On the other hand, as the next steps, we aim to perform the same analysis with methanol extraction in our laboratory because this would allow the comparison of both extracts. However, as a preliminary study, we believe that this study is crucial in terms of foundation for future research.

Conflict of Interest

The authors declare no conflict of interes

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Research Article

A Mutant of *Listeria monocytogenes* Shows Decreased Virulence and Confers Protection Against Listeriosis in Mice

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Abstract

This research was performed to obtain a safe and highly immunogenic *Listeria* strain and evaluate the biological characteristics of the deletion mutant. Based on homologous recombination technology, we constructed a deletion mutant *Lm90-ΔinlB* of *L. monocytogenes*. Meanwhile, we characterized its safety and protective efficacy against listeriosis infection in mice. The results showed that the virulence of *Lm90-ΔinlB* could significantly decrease compared with the parental strain *(Lm90)*. The deletion strain retained hemolytic activity and induced CD^{8+} T cell response comparable to that of *Lm90*. Mice immunized with *Lm90-ΔinlB* were capable of stimulating specific CD^{8+} T cells to the listerial epitopes LLO_{91-99} and $P60_{217-225}$ at levels equivalent to *Lm90*. Importantly, immunization of mice with *Lm90-ΔinlB* displayed good protection against listeriosis. In conclusion, strain *Lm90-ΔinlB* is a vaccine candidate with the potential to be more immunogenic yet considerably less toxic than the parental strain.

Keywords: *Listeria monocytogenes, inlB gene, CD^{8+} T cells, Protective efficacy, Vaccine*

Farelerde Düşük Virulans Gösteren ve Listeriosise Karşı Koruma Sağlayan *Listeria monocytogenes* Mutantı

Öz

Bu araştırma güvenli ve yüksek derecede immunojenik bir *Listeria* suşu elde etmek ve delesyon mutantının biyolojik özelliklerini değerlendirmek için yapıldı. Homolog rekombinasyon teknolojisi kullanılarak, *L. monocytogenes*'in bir delesyon mutantı olan *Lm90-ΔinlB* oluşturuldu. Aynı zamanda, farelerde listeriosis enfeksiyonuna karşı güvenliği ve koruyucu etkinliği belirlendi. Sonuçlar, *Lm90-ΔinlB*'in virülansının, parental suşa *(Lm90)* kıyasla önemli ölçüde azalabileceğini gösterdi. Delesyon mutantı suş hemolitik aktiviteyi korudu ve CD^{8+} T hücre tepkisini *Lm90* ile karşılaştırılabilir düzeyde uyardı. *Lm90- ΔinlB* ile immünize aşılanmış farelerde, *Lm90*'a eşdeğer seviyelerde LLO_{91-99} ve $P60_{217-225}$ listeria epitoplarına spesifik CD^{8+} T hücrelerini uyarabilme kapasitesi mevcuttu. Farelerde *Lm90-ΔinlB* ile aşılanması sonrası listeriyoza karşı iyi bir koruma görülmesi önemli bir bulgudur. Sonuç olarak, *Lm90-ΔinlB* suşu, parental suşundan daha fazla immünojenik ancak önemli ölçüde daha az toksik olması nedeniyle potansiyel bir aşı adayıdır.

Anahtar sözcükler: *Listeria monocytogenes, inlB geni, CD^{8+} T hücreleri, Koruyucu etkinlik, Aşı*

INTRODUCTION

Listeria monocytogenes is a Gram-positive pathogen that can cause listeriosis with gastroenteritis, meningitis and encephalitis [1,2]. As a food-borne pathogen, *L. monocytogenes* can cross the intestinal barrier through intestinal epithelial cells or phagocytes and reach the liver and spleen via the lymph and bloodstream. *L. monocytogenes* multiplies rapidly and finally spreads to the brain through blood circulation. *L. monocytogenes* is known to affect pregnant women, immunocompromised individuals, the young, and the elderly via the oral route [3-5]. *L. monocytogenes* has typical characteristics of intracellular parasitism and intercellular transmission, and could simultaneously cause MHC-I and MHC-II antigen delivery system and stimulate the host to produce strong cellular immune response [6,7]. As cytotoxic T lymphocytes (CTLs) are thought to be an important defense against tumor, virus and intracellular bacterial pathogens, attenuated *L. monocytogenes* has the ability to stimulate this immune response, which has broad clinical relevance. At present, several attenuated *L. monocytogenes* have been successfully used in tumor, virus and other DNA vaccine vectors, and some vaccines have entered phase I and phase II clinical trials [8,9].

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L. monocytogenes has abilities to infect not only phagocytes but also non-phagocytes. The infection process can be divided into four stages: invasion, escape phagocytosis, multiplication and transmission between cells. In the process of infection, each step needs to be done by a specific virulence factor. Internalins are the protein products of a family of virulence-associated genes found in pathogenic *Listeria* spp. Internalin A (InlA) and Internalin B (InlB) encoded by the *inlAB* operon were the first members of this family to be characterized. And the two proteins could play important role in *L. monocytogenes* invasion [10]. Specifically, InlA binds to E-cadherin receptor through its LRR region for invading intestinal epithelial cells and trophoblast cells. InlB not only binds to Met receptor through LRR region, but also binds to receptors of gClqR and GAGs through GW region, thus invading hepatocytes, Vero cells and Hela cells [11,12]. Meanwhile, *L. monocytogenes* strongly induces cell-mediated immune responses. As a result of its cytoplasmic location during infection and its particular advantages as a neonatal vaccine vehicle, *L. monocytogenes* can facilitate a long-term cellular immune response, which makes attenuated strains a focus of attention in vaccine development [9,10]. Previous studies have shown that *L. monocytogenes* tropism into hepatocytes is mediated by the virulence factor InlB. Therefore, deletion of *inlB* is expected to limit liver toxicity. Undeniably, *ΔinlB* mutants display reduced hepatocyte entry during the infection of monocytes as efficiently as wild-type strains [11,12].

In this study, we successfully constructed a live-attenuated vaccine strain, *Lm*90-*ΔinlB*. The biological characteristics of the deletion mutant were evaluated at the molecular, cellular and preliminary animal levels, which lays a scientific foundation for the further study of the Listeria vaccine vector and vaccine.

MATERIAL and METHODS

Bacterial Strains and Cell Lines

Listeria monocytogenes (*Lm*90, serotype 4b), isolated from a sheep with encephalitis in Xinjiang, China, was used in this study. An *inlB* deletion mutant (called *Lm*90-*ΔinlB*) was constructed using *Lm*90 as the parental strain. To achieve stationary phase, all the strains were grown in brain-heart infusion (BHI) agar (Oxoid, Basingstoke, UK) or broth without antibiotics at 37°C. For solid media, agar was added at 1.5% (w/v), and chloramphenicol (Cm) from Sigma was added at a concentration of 10 μg/mL.

MBMEC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 for adhesion, invasion and intracellular growth assays. Cell culture media and reagents were all obtained from Gibco.

Animals and Ethical Concerns

BALB/c mice of 6-8 weeks of age were obtained from the Laboratory Animal Research Institute of the China Academy of Medical Sciences. All mice were housed in the facilities with the relatively constant temperature of 25±2°C and were treated in strict accordance with the Ethical Committee for animal use in Shihezi University. During the experiments, every effort was made to minimize animal suffering.

Construction and Identification of Lm90-ΔinlB

A splicing-by-overlap-extension (SOE) technique was utilized to construct the deletion mutant [13]. The sequences of primers *(Table 1)* were designed using Primer 5.0 software (Premier Inc., Canada) according to the sequence within the *L. monocytogenes* F2365 genome in the GenBank database (accession number: AE017262.2). *L. monocytogenes* genomic DNAs were extracted using the DNA extraction kit (Tiangen Bio, Co.). The 5′ upstream and 3′ downstream homologous arms were amplified by polymerase chain reaction (PCR) using primers P1/P2 and P3/P4, respectively. Then mixed the products in a 1:1 ratio and used SOE PCR technique to achieve the fusion fragment with the primer P1/P4. The fusion fragment was recovered and ligated to pMD19-T cloning vector (TaKaRa Bio, Inc.). The *ΔinlB* fragment and shuttle vector pKSV7 were digested with *Eco*RI and *Hin*dIII (Takara Bio, Inc.), respectively. Then the *ΔinlB* fragment was cloned into pKSV7 to obtain pKSV7-*ΔinlB*, and then the recombinant plasmid was transformed into *L. monocytogenes* competent cells by electroporation [14]. Under the pressure

Table 1. *PCR primers used in the study*

Primers	Sequence (5′--3′)	Restriction Site	Product Length (bp)	Target Gene
P1	CCCAAGCTTGGGGGGTCGCTTGTTACTCCA	*Hin*dIII	339	Upstream sequence of the *inlB* gene
P2	GAAATAGCTTTTCGTAGGATAATCCGTACTAAAATC			
P3	TTTTAGTACGGATTATCCTACGAAAAGCTATT		415	Downstream sequence of the *inlB* gene
P4	CGGAATTCCG TACGCAAACTGGCAAAGC	*Eco*RI		
P5	GGGTCGCTTGTTACTCCA		750	Detecting primers
P6	TACGCAAACTGGCAAAGC			
hly-F	CTGAATTCGGCTGTTACTAAAGAGCAGTTGC		743	*hly*
hly-R	ATGGATCCTTAGCCCCAGATGGAGATATTTCTA			

of chloramphenicol and temperature, the recombinant strain was selected on the basis of methods described previously [15].

Growth Curve and Growth Activity Assays of the Bacteria

Bacterial growth was measured by direct optical density detection at 600 nm and enumeration of the colony-forming units (CFU) in serial dilutions plated on BHI agar.

Specifically, after cultured for almost 16 h, appropriate amount of *Lm*90 and *Lm*90-*ΔinlB* was inoculated into fresh medium at the ratio of 1:100 and cultured at 37°C. Bacterial growth was determined every 2 h by the optical density and CFU.

Cell Culture and Infection Experiments

MBMEC cells were cultivated in 24-well plates in DMEM medium containing 10% heat-inactivated FBS at 37°C under a 5% CO_2 atmosphere. For adhesion, invasion and intracellular growth assays, $2x10^5$ cells were seeded in 24-well tissue culture plates 1 to 2 days before infection. Meanwhile, *L. monocytogenes* were cultured to an optical density about 0.3 at 600 nm. Cell layers were washed with PBS and infected in triplicate with 1 mL of bacteria suspended in medium without FBS for 1 h. Then the culture media were collected, diluted and cultured on BHI agar plates to enumerate the bacteria. During the invasion experiment, the medium was replaced with fresh media without FBS and 100 µL of bacterial culture was added into each well. After 1 h of cultivation, the cells were washed and incubated for different time points with medium containing 100 µg/mL^{-1} of gentamicin (Sigma) to kill the extracellular bacteria. Finally, the cells were lysed in 2 mL of DMEM containing 0.2% Triton X-100 (Amresco Inc., USA) to release intracellular bacteria and then the mixture was diluted and spread on BHI agar plates to count the colonies.

Virulence of Listeria Strains In-vivo

Mice were randomly divided into 2 groups and each group had 5 subgroups (n=5). *L. monocytogenes* were cultured overnight, collected by centrifugation and washed with PBS for 3 times. Then the bacteria were diluted by 10-fold gradient in PBS and the mice were intraperitoneally injected with 100 µL of the bacteria with different dilution to determine the median lethal dose (LD_{50}) of listerial strains. The mice were then observed for 14 days and the LD_{50} was measured. The remaining bacteria were cultured on BHI agar plates for bacterial counts. On the 1st, 3rd, 5th and 7th days after intraperitoneal injection of 0.1 LD_{50}, the livers and spleens of mice were sampled quickly to assay the number of bacteria.

Determination of Hemolytic Activity

Hemolytic activity was assayed as described by Portnoy [16] with some modifications. Listerial strains were cultured for 12 h, then the supernatant was collected and the concentration was adjusted according to the OD600 value. Briefly, two-fold serial dilutions of bacterial culture supernatant were made in PBS [pH 6.0] and 70 µL was added per well. After a 30-min incubation at 37°C, 30 µL of 1% sheep red blood cells was added to the sample and mixed. After an additional 30-min incubation at 37°C, the bacterial hemolytic activity was observed and expressed as the reciprocal of the highest dilution. A negative control comprising PBS only was included in the experiment.

Immunization Procedure

Mice were separated into 3 groups of 15 each. On days 1 and 14, one group of mice was immunized intraperitoneally *(i.p.)* with 0.1 LD_{50} of *Lm*90-*ΔinlB* in a total volume of 200 µL. On the same days, mice in the negative control group were given 200 µL PBS, whereas mice immunized *i.p.* with a sublethal dose of *Lm*90 ($2.0x10^4$ CFU per 200 µL) were considered as a positive control group. On day 35, five mice from each group were euthanized for ELISPOT assay and 10 mice were prepared for challenge assay.

ELISPOT Assay

The enzyme-linked immunospot (ELISPOT) assay was used to determine the levels of the major *L. monocytogenes* antigens, LLO_{91-99} and $P60_{217-225}$. To determine the levels of protective immunity of *Lm*90-*ΔinlB*, T cell responsiveness was analyzed using a standard ELISPOT approach, which was performed according to the protocol in the published papers [17-20].

Challenge Assay

According to the immunization procedure above, 10 mice from each group were challenged with 3.0×10^5 CFU per 200 µL of *Lm*90 via the *i.p.* route on day 35. Three days later, all mice were euthanized, then the spleens and livers were collected, homogenized and cultured to determine the CFU of *L. monocytogenes*. Bacteria were enumerated by plating serial dilutions of organ homogenates on BHI agar and incubating 16 h at 37°C.

Statistical Analysis

One-way ANOVA with post hoc analysis by the Dunn's method was used in this study. Differences with a P-value of <0.05 were considered statistically significant. Differences between groups were analyzed using the Statistical Package for Social Sciences software (SPSS 20.0). Graphs were prepared using GraphPad Prism 6.0 graphing software.

RESULTS

Construction of the inlB Deletion Mutant Lm90-ΔinlB

Based on the homologous recombination technology described in the Materials and Methods, we constructed deletion mutant *Lm*90-*ΔinlB*. A single band of 750 bp was amplified from the *Lm*90-*ΔinlB* strain and a good genetic

stability was observed during continuous passage to 20 generations *in vitro (Fig. 1)*, which indicated that *inlB* gene had been deleted from the *Lm*90 genome. With sequencing techniques, we further confirmed that the *inlB* gene had been deleted and had genetic stability *(Fig. 2)*.

InlB did not Affect the Growth of L. monocytogenes

In the study, we measured the growth of *Lm*90 and *Lm*90-*ΔinlB* to examine whether the *inlB* deletion affected the growth of *L. monocytogenes*. The results showed that there were no obvious differences between *Lm*90 and *Lm*90-*ΔinlB (Fig. 3-A)*. In addition, the number of CFU between the two strains also showed no obvious difference *(Fig. 3-B)*. The results in this section indicated that the *inlB* deletion did not affect the growth of *L. monocytogenes*.

Adhesion and Invasion Characteristics of the Deletion Mutant

In the MBMEC cell adhesion assay, *Lm*90 exhibited 4.63% adhesion compared with only 2.52% for *Lm*90-*ΔinlB*. Similarly, in the MBMEC cell invasion assay, *Lm*90 exhibited 0.37% invasion compared with only 0.23% for *Lm*90-*ΔinlB (Fig. 4-A)*. In addition, the number of viable intracellular bacteria for *Lm*90-*ΔinlB* was significantly lower than that of *Lm*90 ($P<0.05$) *(Fig. 4-B)*.

The Virulence of the Deletion Mutant was Reduced in Mice

A notable reduction in virulence was observed with the deletion mutant compared with the parental strain, as shown in *Table 2*. The LD_{50} values for *Lm*90 and *Lm*90-*ΔinlB* in experimental mice were $10^{4.60}$ and $10^{7.38}$ CFU, respectively. The LD_{50} value of *Lm*90-*ΔinlB* was increased by 2.78 orders of magnitude, indicating that *L. monocytogenes* virulence significantly ($P<0.05$) decreased with the lack of *inlB* gene. Furthermore, the number of viable bacteria in the liver and spleen of mice infected with *Lm*90-*ΔinlB* was significantly lower than that of mice infected with *Lm*90 ($P<0.05$) *(Fig. 5)*. In summary, the survival and proliferation of *L. monocytogenes* on days 1-7 post-infection significantly decreased due to the deletion of the *inlB* gene and the virulence of *Lm*90-*ΔinlB* was decreased. Taken together, these results indicated a significant improvement in the safety of *Lm*90-*ΔinlB*.

The Deletion Mutant Retains Hemolytic Activity

The major *L. monocytogenes* antigen listeriolysin O (LLO) confers the unique adjuvant characteristics of *L. monocytogenes* of being able to generate a Th1 immune response. Hemolysis activity results of the *L. monocytogenes* strains were presented in *Fig. 6* and the hemolysis titer of *Lm*90-*ΔinlB* reached 2^5, which was a little lower than that of *Lm*90 (2^6). Therefore, we concluded that *Lm*90-*ΔinlB* had good hemolytic activity.

The Deletion Mutant Induces an Antigen-specific CD8⁺ T Cell Response Similar to the Parental Strain

In the light of immunization procedure in the methods, all experimental mice were vaccinated with primary inoculation

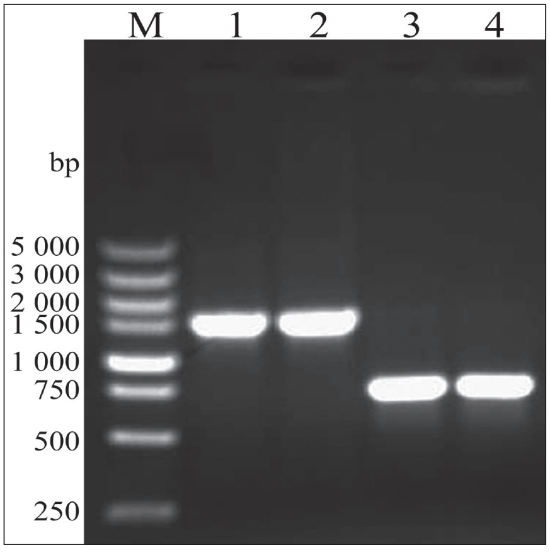

Fig 1. Identification of recombinant strain *Lm*90-*ΔinlB* by polymerase chain reaction. M: DNA marker. 1,2: *Lm*90 used as a positive control. 3: pKSV7-*ΔinlB* used as a positive control. 4: PCR products from *Lm*90-*ΔinlB*

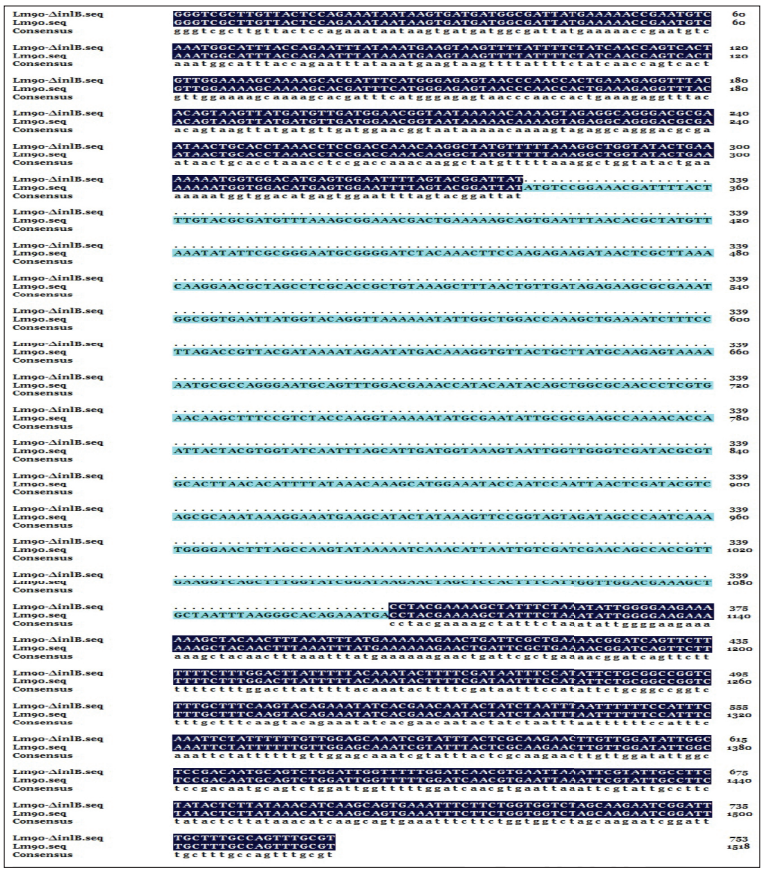

Fig 2. Comparison of sequencing result of amplified products of *Lm*90-*ΔinlB* with the corresponding sequence of *Lm*90

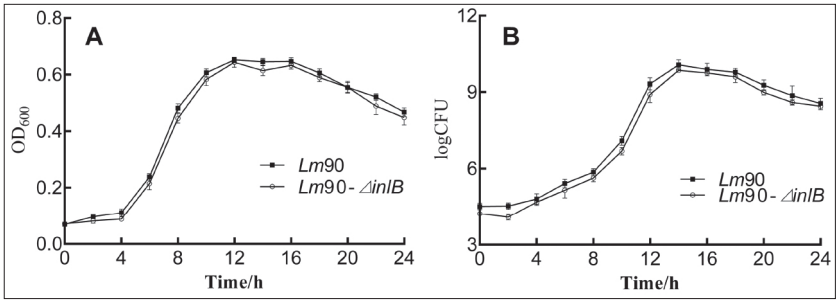

Fig 3. A: Growth curves of *Lm*90 and *Lm*90-*ΔinlB*. Data are from experiments repeated at least three times. B: CFU determination of *Lm*90 and *Lm*90-*ΔinlB*, results are shown as $\log_{10}$ CFU. Bars represent the mean ± SD. No statistically significant differences in growth were observed between strains

Fig 4. A: Results of adhesion and invasion assay in MBMEC cells. * $P<0.05$ according to Kruskal-Wallis one-way ANOVA, with post hoc comparison using Dunn's method. Differences are shown relative to *Lm*90. Error bars represent the mean ± SEM; B: Intracellular growth of *Lm*90 and *Lm*90-*ΔinlB* in MBMEC cells using a gentamicin protection assay. No statistical significance was observed

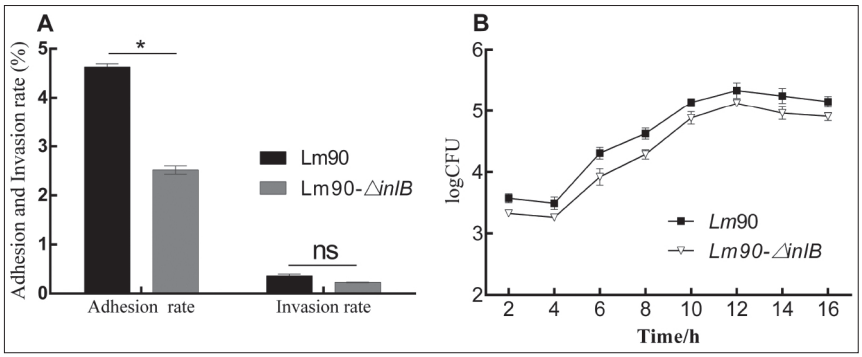

Table 2. *The 50% lethal doses of Lm90 and Lm90-ΔinlB in mice*

Group	*Lm*90			Group	*Lm*90-*ΔinlB*		
	Dose/CFU	Mortality	LD_{50}		Dose /CFU	Mortality	LD_{50}
1	2.0×10^{8}	5/5	$10^{4.60}$	1	3.0×10^{8}	5/5	$10^{7.38}$
2	2.0×10^{7}	5/5		2	3.0×10^{7}	2/5	
3	2.0×10^{6}	5/5		3	3.0×10^{6}	1/5	
4	2.0×10^{5}	4/5		4	3.0×10^{5}	0/5	
5	2.0×10^{4}	2/5		5	3.0×10^{4}	0/5	

CFU, colony-forming unit; LD_{50}, 50% lethal dose

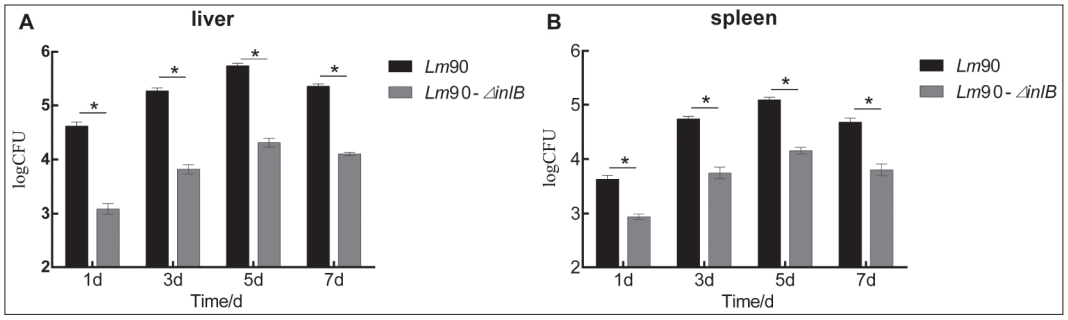

Fig 5. The bacterial count of *Lm*90 and *Lm*90-*ΔinlB* in (A) the liver and (B) the spleen of mice infected by intraperitoneal injection. The data represent three repeats of serial dilutions from one injection. CFU, colony-forming unit. Bars represent the mean ± SEM, and asterisks indicate statistical significance ($P<0.05$)

on day 1 and a booster on day 14. Using a standard ELISPOT method, T-cell responsiveness to $LLO_{91\text{-}99}$ or $P60_{217\text{-}225}$ of mice were examined at day 35 and the results were shown in *Fig. 7*. Mice immunized with *Lm*90-*ΔinlB* elicited $LLO_{91\text{-}99}$-specific $CD8^+$ T cells at levels equivalent to *Lm*90. In the negative control group, no induction of LLO-specific $CD8^+$ cells was observed. Correspondingly, compared with the negative control group, the induction of $P60_{217\text{-}225}$-specific $CD8^+$ T cells showed a statistically significant ($P<0.01$) in the mice immunized with *Lm*90-*ΔinlB* or *Lm*90, respectively.

IP Vaccination with Deletion Mutant (Lm90-ΔinlB) Provides Protection Against Subsequent Parental Strain (Lm90) Challenge in Mice

According to immunization procedure as referred above,

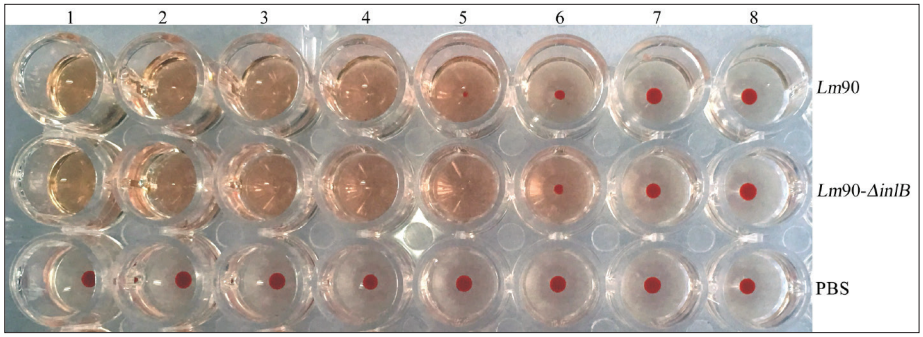

Fig 6. Hemolysis activity test result of mutant strains and parent strain. Culture supernatants were incubated with sheep erythrocytes at 37°C in two-fold serial dilutions (wells 1-8). The mutant strain of *Lm*90-*ΔinlB* had hemolysis titers of 2^5, while the parental strains were 2^6

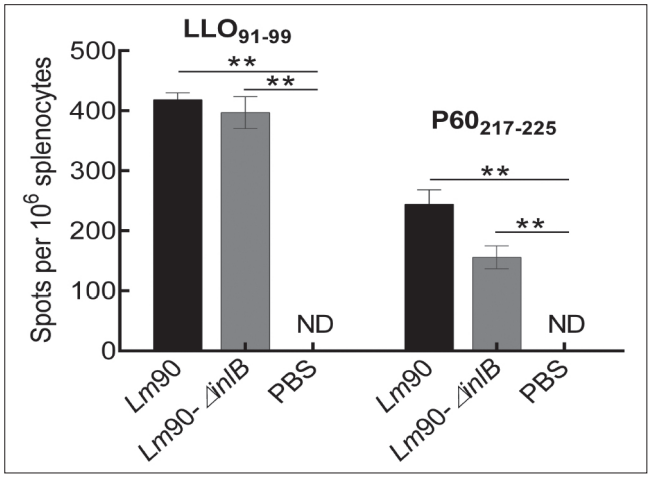

Fig 7. ELISPOT assay. Vaccination injections were given on days 1 and 14 for all murine groups (n=5). Each spot represents one IFN-γ-secreting $CD8^+$ T cell specific to the listerial epitopes LLO_{91-99} and $P60_{217-225}$. Bars represent the mean ± SEM, and asterisks indicate statistical significance (*P*<0.01) compared with the negative control group to which PBS was administered

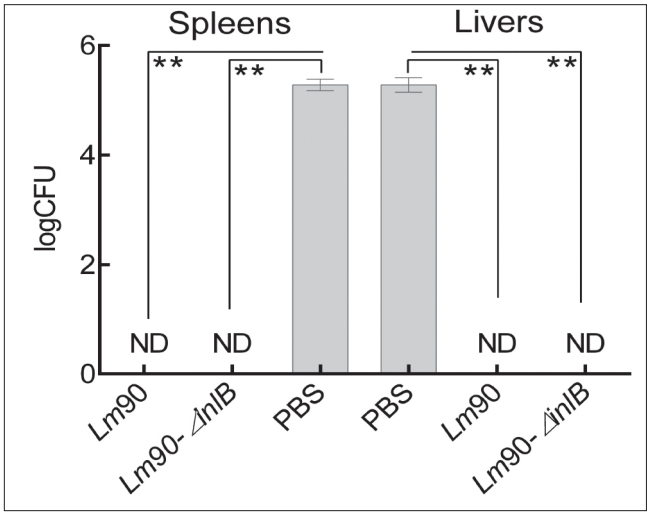

Fig 8. Challenge assay. All murine groups (n=10) were vaccinated via *i.p.* injection on days 1 and 14 prior to challenge with *Lm*90 on day 35. Mice were euthanized 3 days post-challenge and the c.f.u. in the livers and spleens were enumerated. Bars represent the mean ± SEM, and asterisks indicate statistical significance (*P*<0.01) compared with the negative control group to which PBS was administered

the remaining 10 mice from each group were challenged with *Lm*90 on day 35 via the *i.p.* route. Then all mice were euthanized 3 days post-challenge and the spleens and livers were harvested for bacterial counts. By comparison with the PBS-treated group, the mice immunized with *Lm*90-*ΔinlB* showed a protection from listerial challenge and the result was similar to that of *Lm*90 group (*P*<0.01). And the bacterial loads in the organs showed no significant difference between the *Lm*90-*ΔinlB* and *Lm*90 groups (*P*>0.05). On the other hand, the organs of mice in the PBS-treated group had a great deal of bacterial loads after 3 days *(Fig. 8)*.

DISCUSSION

Listeria monocytogenes is capable of provoking strong cell-mediated immune responses and has been widely studied as a model intracellular pathogen. Attenuated listerial strains are often used to convey vaccine antigens since *L. monocytogenes* can induct of major histocompatibility complex (MHC) class I-restricted immune responses [21,22].

In our study, we successfully constructed deletion mutant *Lm*90-*ΔinlB* of *L. monocytogenes*. A product of 750 bp was generated by PCR amplification using recombinant *Lm*90-*ΔinlB* with primers P5/P6. When cultured at 37°C, no significant difference in growth between *Lm*90 and *Lm*90-*ΔinlB* was observed (*P*>0.05, *Fig. 3-A,B)*. It is known that *L. monocytogenes* infects both phagocytes and non-phagocytic cells. The research of Gaillard et al.[23] is valuable to our understanding of the function of internalins. In their study, *L. monocytogenes* was shown to enter into non-phagocytic cells mediated by InlA and InlB. During *L. monocytogenes* infection, InlA promotes listerial uptake into human intestinal epithelial cells (IECs) by means of binding to E-cad [23]. Comparably, InlB has the property to promote invasion into hepatocytes by means of binding to InlB receptor, c-Met. InlB activates c-Met route in the process of invasion and makes easy to enter into cells by inducing actin polymerization [24]. Furthermore, gC1qR and glycosaminoglycans also bind to InlB and promote invasion. Here we focused on InlB and an *inlB* gene deletion strain *(Lm90-ΔinlB)* was constructed in *Lm*90. The virulence of *L. monocytogenes* in mice significantly decreased with the absence of *inlB* and its proliferation in the organs of mice also decreased. In cell adhesion and invasion experiments, the virulence of *L. monocytogenes* significantly decreased with the absence of *inlB* and the

results were in line with previous observation studied by Pentecost et al.[25]. In a murine model, a *ΔinlB* mutant of *L. monocytogenes* produces fewer bacterial counts in the liver [26,27]. Chiba et al.[28] constructed *ΔinlB-Lm* using the 10403s strain and *ΔinlB-Lm* showed less efficiency in invading spleen. In addition, on day 3 after infection, the loads of *ΔinlB-Lm* in various organs were significantly smaller than those of wild-type *L.monocytogenes*. In our study, we also confirmed that the loads of *Lm*90-*ΔinlB* in spleen or liver were significantly smaller than those of the parental strain *(Lm*90). To conclude this section, the results in our study clearly indicated that the importance of *inlB* in the role of regulating virulence and further studies should seek to clarify the involvement of this *L. monocytogenes* virulence factor in pathogenesis.

L. monocytogenes infection induces robust $CD8^+$ T cell responses, which play a critical role in resolving *L. monocytogenes* during primary infection and provide protective immunity against re-infection. It has been shown that both LLO and P60 are major antigen in the protective response against *L. monocytogenes* [29-31]. According to the studies provided by Yamamoto et al.[32] and Kono et al.[33], the secreted protein LLO encoded by the *hly* gene is prominent in generating a Th1 immune response. LLO has been shown to be processed very efficiently into peptides that are presented by MHC class I molecules. LLO_{91-99} is an immunodominant epitope that induces $CD8^+$ CTLs, which protect *in vivo* against *L. monocytogenes* infection and confer significant anti-Listeria immunity on naive mice upon passive transfer [34]. The P60 protein is encoded by the *iap* (for invasion-associated protein) gene [35] and has a notable induction effects on $CD8^+$ T cell response [7]. It is noteworthy that several studies have used live bacterial vectors, such as *Salmonella typhimurium* (*S. typhimurium*) and *Lactococcus lactis*, expressing LLO and/or P60 for vaccination against listeriosis [36,37]. Here we tested the bacterial hemolytic activity of *Lm*90-*ΔinlB* and *Lm*90, and no significant differences were detected between the two strains *(Fig. 6)*. We also showed that mice immunized with *Lm*90-*ΔinlB* could stimulate specific $CD8^+$ T cells against LLO_{91-99} and $P60_{217-225}$ at levels equivalent to *Lm*90. These data indicated that *Lm*90-*ΔinlB* was capable of inducing a powerful *Listeria*-specific T cell response and considerably higher than that of mice inoculated with PBS *(Fig. 7)*.

Previous work, as well as work described by McLaughlin et al.[38], mice immunized with *ΔfrvA* following two *i.p.* immunization doses on days 1 and 14 elicited LLO_{91-99} specific $CD8^+$ T cells at levels comparable with the wild type *L. monocytogenes* on day 35. In our ELISPOT assay, we found that *Lm*90-*ΔinlB* was able to induce $CD8^+$ T cell response effectively, and a challenge assay was carried out to examine whether *Lm*90-*ΔinlB* could be used as a vaccine against listeriosis in mice. The results showed that the mice undergone prime and boost vaccinations, which proved a protective effect against challenge with *Lm*90 *(Fig. 8)*.

In conclusion, our study suggested that *L. monocytogenes* virulence, adhesion, invasion and proliferation were significantly related to the absence of *inlB*. Moreover, *Lm*90-*ΔinlB* retains potent immunogenicity and exhibits significantly decreased virulence compared with parental strain *Lm*90. Our data also demonstrated that the activity of CTLs induced by *Lm*90-*ΔinlB* extremely increased. Importantly, immunization of mice with *Lm*90-*ΔinlB* offered complete protection against listeriosis. This suggests that strain *Lm*90-*ΔinlB* is a vaccine candidate that is more immunogenic yet considerably less toxic than the parental strain and may be used in future work.

Author Contributions

GY and JJ designed the overall study especially the immunization procedure and challenge assay. JR and MY performed all the experiments with assistance and advice from GY, JJ and PW. Laboratory data analysis was performed by MY. The manuscript was written by JR and MY, and reviewed by the co-authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest concerning this work.

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Research Article

The Effects of Calcium Aluminate and Calcium Silicate Cements Implantation on Haematological Profile in Rats

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Abstract

The objective of this study was to evaluate potential unfavourable side effects, especially on the blood corpuscles, of experimental calcium aluminate and calcium silicate cements that are applied subdermally or directly to tooth pulp. In the study, fifty-four Wistar rats were separated into two study groups (n=27 in each group). The effects of the dental cements on haematological parameters were observed three times (after 7, 15 and 30 days): erythrocytes, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, leukocytes, leucogram and poikilocytic erythrocyte forms. There were no statistically significant differences in the total number of leukocytes and percentage results of lymphocytes, and neutrophils. The study recorded lower numbers of erythrocytes, with no connection with the type of material applied to the dental pulp (First study group). Significant differences in results for the second study group were recorded on days 7-15 and 15-30 of the experiment in relation to both types of implanted cements. In the second study group there were significant differences related to both types of implanted cements, in the same periods. Lower values of erythrocytes, haemoglobin and haematocrit indicated the existence of hypochromic anaemia caused by the damaging influence of calcium aluminate and calcium silicate. Research showed that in both study groups, normocytic hypochromic anaemia existed, along with modest to expressed distribution of anulocytes and stomatocytes. According to the results of the research, the negative impact of application of Ca-silicate was recorded and was related to the appearance of hypochromic anaemia with poikilocytic forms of erythrocytes.

Keywords: *Calcium aluminate, Calcium silicate, Rat, Haematological parameters, Poikilocytic erythrocytes forms*

Kalsiyum Alüminat ve Kalsiyum Silikat Sement İmplantasyonunun Ratlarda Hematolojik Profil Üzerine Etkileri

Öz

Bu çalışmanın amacı, deneysel olarak subdermal veya direkt olarak diş pulpasına uygulanan kalsiyum alüminat ve kalsiyum silikat sementlerin, özellikle kan kürecikleri üzerindeki potansiyel olumsuz yan etkilerini değerlendirmektir. Çalışmada, elli dört Wistar rat iki çalışma grubuna ayrıldı (her grupta n = 27). Dental sementlerin hematolojik parametreler üzerindeki etkileri eritrositler, hemoglobin, hematokrit, ortalama korpüsküler volüm, ortalama korpüsküler haemoglobin, ortalama korpüsküler hemoglobin konsantrasyonu, lökositler, lökogram ve poikilositik eritrosit formlarını içerecek şekilde üç kez (7, 15 ve 30 gün sonra) gözlemlendi. Total lökosit sayısı ile lenfosit ve nötrofillerin yüzde değerlerinde istatistiksel olarak anlamlı bir fark yoktu. Çalışma, eritrosit sayısında diş pulpalarına uygulanan materyal tipiyle hiçbir bağlantısı olmayan bir azalmayı ortaya koydu (Birinci çalışma grubu). İkinci çalışma grubu için elde edilen sonuçlarda, 7-15 ve 15-30. günlerde her iki sement implant tipiyle ilişkili olan anlamlı farklılıklar saptandı. İkinci çalışma grubunun aynı periyotlatında her iki sement implant türü ile ilgili önemli farklılıklar vardı. Düşük eritrosit, hemoglobin ve hematokrit değerleri, kalsiyum alüminat ve kalsiyum silikatın zararlı etkisinden kaynaklanan hipokromik aneminin varlığını gösterdi. Bu araştırma ile her iki çalışma grubunda da anülositlerin ve stomatoitlerin orta dereceli ekspresyonunu gösteren normositik hipokromik anemi varlığı belirlendi. Araştırma sonuçlarına göre, Ca-silikat uygulamasının olumsuz etkisi saptandı ve bu durum eritrositlerin poikilositik formları ile birlikte görülen hipokromik anemi ile ilişkiliydi.

Anahtar sözcükler: *Kalsiyum alüminat, Kalsiyum silikat, Sıçan, Hematolojik parametreler, Poikilositik eritrosit formları*

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INTRODUCTION

Biomaterials are defined as materials designed to successfully replace the corresponding biological function in close contact with living tissue, after they have been implemented in the body of an animal or human. The biocompatibility of some biomaterials is evident. They show excellent characteristics for application in the field of surgery [1,2]. In contact with biomaterial, tissue should not exhibit cytotoxic, genotoxic, mutagenic or allergic reactions [3-5]. If changes occur on the surrounding tissue, they are in the form of inflammation, necrosis, infiltration, encapsulation or some other reaction by the tissue [6].

Some implanted biomaterials have a partially adverse impact on leukocytes in peripheral blood, in the form of the occurrence of toxic granules in the cytoplasm [7]. Many researchers have studied the biocompatibility of calcium silicate cements and, in the last decade, calcium aluminate cements [5,6,8,9]. There is a growing interest in nanomaterials since, among other things, they have the possibility to overcome the problems associated with solubility and stability, and to enable medicine to be released into the desired tissue [10].

Using nanotechnology, in combination with the hydro-thermal sol-gel method and self-inflicting waves method, two new experimental biomaterials based on calcium aluminate and calcium silicate were synthesized. Expectations are that these biomaterials will overcome the existing disadvantages of popular calcium silicate cements such as MTA: long bonding time, and the granular consistency of the fused material.

In dental research, the most used animal for laboratory tests is rat [11-13]. A literature review revealed many studies where rats were used to follow the effects of different substances on specific tissues [12,14]. In contrast, not so many authors, in their evaluation of the toxicity or biocompatibility of implanted materials, have investigated their influence on haematological parameters [15,16].

It is evident that monitoring and analysis of haematological parameters is one in the series of important segments of every clinical examination of a sick animal. Haematological analysis contributes to determining potential disease risk, or exclusion of possible diseases, following the disease's progress and path, as well as therapy success and outcome of treatment [11,13]. On the other hand, using haematological parameters as a tool for evaluation of biomaterial toxicity would create the possibility of quick and efficient selection of appropriate implantation material.

The objective of this study was to evaluate the potential influence on blood corpuscles of experimental calcium aluminate and calcium silicate cements, applied sub-dermally or by direct coverage of the tooth pulp of rats. In order to attain the most accurate results, a number of haematological parameters were analysed. New experimental biomaterials were used to establish their purposefulness for veterinary dentistry and medicine, as well as their unfavourable side effects on haematological profile.

MATERIAL and METHODS

Ethics Committee Approval

This study was approved by the Ethics Committee of the University Clinical Center in Banja Luka under registration number 01-9-192.2/15, Bosnia and Herzegovina.

Testing Materials

The materials used for testing are calcium aluminate systems: a $CaO{\bullet}Al_2O_3 + CaCO_3 + Bi_2O_3$, mixture called ALBO-MCCA obtained by mixing $CaCO_3$ and Bi_2O_3, and BaS04 with calcium aluminate phase with a ratio of 2:2:1. Finally, water was added to the mixture in a 1:2 ratio, to create cement paste. The second material used was calcium silicate (CS): 60% of the total quantity was β-C2S and C3S phase, with added components: 20% calcium carbonate ($CaCO_3$) and 20% $BaSO_4$ (Merck, Germany) [5,6,17].

Animals

A total of 54 rats, Wistar breed, aged 10-11 weeks, average weight 265-280 g, were used in this study. The rats had free access to food and water, and a 12-h light and darkness schedule. The air temperature was between 20 and 23°C, with 60%±10% air humidity.

General Experimental Procedure and Study Groups

The tested animals (n=54) were separated into two study groups. The first study group consisted of 27 rats subjected to covering the dental pulp with dental cements: Ca-aluminate and Ca-silicate.

Within the first study group, and in its subgroup (A), nine rats had Ca-aluminate administered onto the dental pulp, whereby the pulp was covered directly.

In the subgroup (B), the nine rats had Ca-silicate administered onto the dental pulp whereby the pulp was covered directly.

The remaining 9 rats from the first experimental group represented the control group (A-B).

The other study group of rats also consisted of 27 rats that underwent implantation of sterile polyethylene tubes subdermally on each side of the spine. Polyethylene tubes were used with two types of different fillings: Ca-aluminate and Ca-silicate.

Within the second study group, in its subgroup (C), a sterile polyethylene tube was implanted subdermally on each side of the spine in nine rats, filled with Ca-aluminate.

In subgroup (F), a sterile polyethylene tube containing Ca-silicate was implanted subdermally on each side of the spine in nine rats.

The remaining 9 rats from the second experimental group represented the control group (C-F).

Surgical Procedures

Before the surgical procedure of subdermal implementation of polyethylene tubes, general anaesthesia was performed using intra muscular ketamine, 90 mg/kg of body weight (Ketamine HCl Injection USP) Rotexmedica-Germany, in combination with Xylazin HCl 5 mg/kg of body weight (2% Xylazin, Cp Pharma, Bergdorf, Germany). The duration of anaesthesia was around one hour. Places where application was planned were prepared by removing animal hair and cleaning the area with antiseptic (10% betadine). The polyethylene tubes were inserted into the animals in subgroups C and F as described above.

During the dental procedure, sterile instruments were used including the plates where the material was prepared. The location of application was relatively dry due to the use of absorbent cotton and a saliva pump. Cavities in the first upper molar on the left and right sides of each experimental animal from subgroups A and B were prepared. Trepanation of the pulp cavum top and placement of the cement directly on the pulp was performed. Before application of the cement, the procedure of total anaesthesia was used identical to study subgroups C and F. Cavities were later restored using composite (Tetric Ceram, Ivoclar Vivadent, Schaan Lichtenstein).

Postoperative Treatment

After surgical treatment, the animals were placed separately in cages, with one animal per cage in a strictly controlled environment, with a controlled diet and daily care. Rats from control group A-B were able to consume mashed feed mixture ad libitum, which regarding nutritive values completely responded to the feed consumed by the rats in experimental subgroups A and B. Rats in control groups A-B and C-F were from the same vivarium, hatch, breed and age as the rats in the study groups.

Hematological Procedure

In all rats, the vena coccigee was punctuated at three time periods: 7, 15 and 30 days after surgical procedures. During punctuation, the principles of asepsis and antisepsis were regarded. Punctuated blood was deposited in hematological vacutainers with EDTA anticoagulants.

To estimate the haematological parameters of the peripheral blood, a haematological numerator "Celltac MEK-6450" was used. The parameters that were analysed included: HGB (g/dL), HCT (%), MCV (fl), MCH (pg) i MCHC (g/dL), number of RBC (10^{12}/L) PLT (10^{9}/L) and WBC (10^{9}/L).

Microscopic Examination of Peripheral Blood

Blood smears were processed using standard technical laboratory procedures [18]. On every original coloured smear, 2000 RBC were counted and analysed using a binocular light microscope, Motic Type 102M, 1000x magnification. Poikilocytes were defined according to the standard methodology. Counting was limited to representative one-layer fields in which around half of the erythrocytes were in contact, without overlapping [18-21]. Most representative fields were electronically recorded using Motic Images Plus 2.0 software.

The number and type of poikilocytes was recorded as percentages of RBC. Poikilocytosis was classified semi-quantitatively according to similar research, following the criteria: non-existing (0%), rare (0.05-0.5%), mild (>0.5-3%), modest (>3-10%), or expressed (>10%) [18].

Leukocytes cells were differentiated and numbers were presented in percentages after the analysis of 2000 of them in each animal in the experiment.

Statistical Data Analysis

The mean and standard deviation of the data were determined, after which statistical analysis was performed using Minitab 19® [22]. ANOVA Two-way and post hoc Tuckey test were used to detect whether there were significant differences between the groups and treatments.

RESULTS

Lower values of RBC in the two observed groups (7th and 30th day) were recorded after covering of the pulp by calcium aluminate and calcium silicate compared with their controls *(Table 1)*. Recorded values were below the physiological limits for rats [23]. No statistically significant differences between the average values of the experimental and control groups were recorded. The Post Hoc Tukey test showed the significant differences in RBC between the average values on the 7th and 15th days ($P<0.05$) and the 15th and 30th days ($P<0.05$), after subcutaneous implantation of calcium aluminate and calcium silicate. Also regarding HGB, the ANOVA results showed that there was a statistically significant difference related to the variable Day. However, that difference did not show after performing the Post Hoc Tukey test *(Table 2)*.

After performing Post Hoc Tuckey test we can see that there is significant difference between the means of control and subgroup A and control and subgroup B in the results for MCHC *(Table 1)*. The Post Hoc Tukey test showed a significant difference in MCHC between the means of the control and subgroup C, and the control and subgroup F *(Table 2)*.

The Post Hoc Tukey test showed a significant difference in HCT between the means of the 7th and 15th days ($P<0.05$) and the 15th and 30th days ($P<0.05$) *(Table 2)*.

Table 1. *First study group: mean and standard deviation of haematological parameters after direct coverage of pulp with calcium aluminate (A) and calcium silicate (B)*

Subgroup	Day	RBC	HGB	MCV	MCHC	MCH	HCT	PLT	WBC
A	7	5.70±1.58	10.36±24.58	53.76±2.41	34.10±0.00	18.33±0.80	30±0.07	500.67±394.65	8.50±2.85
	15	9.10±1.67	14.83±21.38	49.26±2.97	33.50±1.00	16.50±0.98	44±0.06	545.67±317.97	9.26±1.88
	30	6.91±0.95	12.65±16.26	53.45±0.07	34.250±3.53	18.30±0.14	36±0.05	594.00±45.25	5.95±0.35
B	7	7.77±0.00	12.50±0.00	48.00±0.00	33.50±0.00	16.10±0.00	37±0.00	1171.00±0.00	9.80±0.00
	15	8.13±1.31	13.80±8.66	51.96±5.50	33.00±6.08	17.16±1.85	41±0.02	695.33±174.90	14.36±6.80
	30	6.48±2.28	11.46±36.11	52.96±4.16	33.70±6.08	17.80±1.05	34±0.10	427.67±121.02	7.80±3.67
Control A-B	7	8.28±0.54	14.83±13.87	51.4±2.15	310.0±17.32	18.33±0.81	0.43±0.01	670.67±100.32	9.84±1.36
	15	8.89±0.39	13.73±2.52	52.90±1.08	310.00±20.00	16.50±0.98	0.44±0.03	630.67±226.29	9.27±1.89
	30	9.18±0.83	13.26±5.03	52.97±2.84	323.33±25.17	17.20±0.89	0.43±0.01	832.33±238.12	7.11±0.88
Reference Ranges		7.34-8.85 [23]	14.7-17.3 [23]	46-57 [24]	31.3–34.4 [23]	18.6-20.7 [23]	44.9-51.7 [23]	804-1282 [24]	6.63-20.35 [23]
P	group	0.074	0.306	0.700	0.005**	0.525	0.083	0.233	0.334
	day	0.116	0.187	0.434	0.408	0.141	0.078	0.417	0.068
	day x group	0.283	0.200	0.375	0.955	0.297	0.318	0.122	0.736

Subgroup A- *direct coverage of pulp with calcium aluminate in both teeth;* ***Subgroup B-*** *direct coverage of pulp with calcium silicate in both teeth*

Table 2. *Second study group: mean and standard deviation of haematological parameters after subdermal implementation of calcium aluminate (C) and calcium silicate (F) polyethylene tubes on both sides of spine*

Subgroup	Day	RBC	HGB	MCV	MCHC	MCH	HCT	PLT	WBC
C	7	5.69±1.97	10.56±38.28	53.86±0.15	34.30±8.71	18.50±0.43	30±0.10	902.00±302.37	5.76±3.23
	15	9.84±0.74	17.30±18.19	51.43±1.55	34.13±7.63	17.56±0.56	50±0.05	548.33±161.86	9.06±3.69
	30	6.15±1.40	11.25±19.09	53.50±2.40	34.40±4.24	18.40±1.13	32±0.06	423.00±103.23	5.90±2.26
F	7	7.95±1.18	14.25±21.92	52.30±0.42	34.25±0.70	17.95±0.07	41±0.06	698.00±186.67	13.15±3.18
	15	9.25±1.22	16.53±18.00	52.96±1.30	33.86±1.15	17.90±0.36	48±0.05	402.00±46.93	6.95±5.34
	30	7.57±0.59	12.50±17.67	50.45±2.33	33.05±4.95	16.65±1.06	38±0.04	599.00±4.24	8.25±0.07
Control C-F	7	8.28±0.73	15.70±4.36	51.93±1.72	323.33±20.82	17.17±0.86	0.44±0.02	955.67±84.23	6.85±0.23
	15	8.13±0.84	13.87±8.98	52.52±0.98	309.83±0.29	16.94±1.23	0.43±0.01	636.97±30.81	8.60±1.67
	30	8.49±0.74	13.86±16.25	51.87±1.11	322.27±1.79	16.89±0.50	0.43±0.01	863.51±81.19	7.43±1.22
Reference Ranges		7.34-8.85 [23]	14.7-17.3 [23]	46-57 [24]	31.3-34.4 [23]	18.6-20.7 [23]	44.9-51.7 [23]	804-1282 [24]	6.63-20.35 [23]
P	group	0.141	0.299	0.353	0.000**	0.025*	0.128	0.008**	0.249
	day	0.010*	0.014*	0.604	0.370	0.369	0.004**	0.001**	0.570
	day x group	0.035*	0.030*	0.139	0.405	0.353	0.043*	0.214	0.147

Subgroup C- *subdermal implementation of calcium aluminate polyethylene tubes on both sides of spine, half a tube;* ***Subgroup F-*** *subdermal implementation of calcium silicate polyethylene tubes on both sides of spine, half a tube*

Regarding PLT, after performing Post Hoc Tuckey test, we saw that there is significant difference between the means of the control and subgroup F. The Post Hoc Tuckey test showed the significant difference in PLT between the means of the 7th and 15th days (P<0.01) and the 7th and 30th days (P<0.05) *(Table 2)*.

There were no statistically significant differences in the total number of WBC *(Table 1, Table 2)* or the percentage results for lymphocytes, immature and mature neutrophils.

The Post Hoc Tukey test showed a significant difference related to the mean values of monocytes between the control and subgroup B (P<0.05). We see a significant difference related to the mean values of monocytes between the control and subgroup C (P<0.05), and the control and subgroup F (P<0.01) *(Table 3, Table 4)*.

The Post Hoc Tukey test showed a significant difference related to the mean values of basophil between the 7th and the 30th days (P<0.05) *(Table 4)*.

Results of Microscopic Examination of Peripheral Blood

Analysis of peripheral blood smears in rats showed the significant presence of anulocytes (hypochromic RBC) with low haemoglobin content *(Fig. 1-e, Fig. 2)*.

According to semi-quantitative classification of poikilocytes [18] there were moderate percentage values of stomatocytes present (>3-10%), as well as the same poikilocytic forms

Table 3. *First study group leucogram (%) mean and standard deviation after direct coverage of the pulp with calcium aluminate and calcium silicate*

Subgroup	Days	Mature Neutrophyl	Immature Neutrophyl	Basophils	Acidophils	Lympohocites	Monocyte
A	7 (n=3)	22.00±12.16	2.20±1.05	2.00±0.34	0.66±0.57	70.03±13.19	0.66±0.57
	15 (n=3)	9.00±8.18	1.66±2.88	0.00±0.00	0.33±0.57	85.66±12.05	0.33±0.57
	30 (n=3)	10.00±2.00	1.33±1.15	0.00±0.00	1.00±1.00	82.00±2.00	2.00±0.00
B	7 (n=3)	32.66±3.21	0.66±0.57	0.33±0.57	0.66±0.57	61.33±6.11	0.33±0.57
	15 (n=3)	23.26±20.43	3.20±2.88	0.00±0.00	2.00±1.00	64.76±18.75	1.33±0.57
	30 (n=3)	12.33±5.13	1.33±0.57	0.00±0.00	0.33±0.57	82.66±6.50	1.00±0.00
Control	7 (n=3)	17.00±5.41	0.67±0.76	0.50±0.50	2.00±1.00	77.67±6.81	2.17±1.61
	15 (n=3)	14.5±7.05	1.00±0.00	0.23±0.25	0.50±0.50	79.07±12.57	2.00±1.00
	30 (n=3)	17.67±4.16	1.83±1.04	0.33±0.58	1.00±1.00	75.33±5.13	1.83±1.04
Reference Ranges		3.5-18.7% [23]	-	0.2-0.6% [23]	0.0-0.6% [23]	75.8-92.9% [23]	0.5-3.4% [23]
P	day	0.062	0.572	0.238	0.674	0.133	0.355
	group	0.130	0.673	0.197	0.409	0.146	0.018*
	day x group	0.362	0.367	0.992	0.032*	0.210	0.164

***Subgroup A**- direct coverage of pulp with calcium aluminate in both teeth; **Subgroup B**- direct coverage of pulp with calcium silicate in both teeth*

Table 4. *Second study group leucogram (%) mean and standard deviation after subdermal implementation of calcium aluminate and calcium silicate polyethylene tubes on both sides of spine*

Subgroup	Days	Mature Neutrophyl	Immature Neutrophyl	Basophils	Acidophils	Lympohocites	Monocyte
C	7 (n=3)	13.16±6.52	1.00±1.00	0.33±0.57	1.33±1.52	79.66±5.75	1.50±0.86
	15 (n=3)	15.33±5.85	1.00±1.00	0.00±0.00	1.66±1.52	78.66±7.57	0.33±0.57
	30 (n=3)	21.33±1.52	2.00±0.00	0.66±0.57	0.83±1.44	71.00±3.00	1.83±1.04
F	7 (n=3)	11.68±2.06	1.58±1.50	1.68±0.65	2.26±1.16	76.75±1.61	1.25±0.66
	15 (n=3)	16.66±10.40	0.76±0.40	0.86±0.23	0.00±0.00	78.80±10.92	0.76±0.40
	30 (n=3)	12.33±6.65	1.33±1.52	0.00±0.00	0.00±0.00	83.66±5.85	0.33±0.57
Control	7 (n=3)	20.00±2.65	1.50±0.50	0.67±0.58	0.50±0.50	74.50±1.32	2.17±0.76
	15 (n=3)	19.71±1.69	1.00±1.00	0.27±0.35	1.00±1.00	76.42±0.62	2.15±0.05
	30 (n=3)	20.03±1.91	0.68±0.79	0.17±0.29	1.00±0.00	75.88±2.13	2.24±1.25
Reference Ranges		3.5-18.7% [18]	-	0.2-0.6% [18]	0.0-0.6% [18]	75.8-92.9% [18]	0.5-3.4% [18]
P	day	0.475	0.613	0.016*	0.307	0.892	0.312
	group	0.061	0.628	0.036*	0.516	0.255	0.003**
	day x group	0.444	0.475	0.011*	0.109	0.173	0.239

***Subgroup C**- subdermal implementation of calcium aluminate polyethylene tubes on both sides of spine, half a tube; **Subgroup F**- subdermal implementation of calcium silicate polyethylene tubes on both sides of spine, half a tube*

after thirty days' treatment with calcium aluminate.

Conical shaped RBC with a central dent, or a uniconical shape is called a stomatocyte. As mentioned before, their presence was shown *(Fig. 1-a,c,d; Fig. 2)*.

On the 7th and 15th days, all experimental groups, in both tissues and those treated with both dental materials showed a "mild" appearance of stomatocytes (>0.5-3%) *(Fig. 2)*. The presence of spherocytes is classified as "moderate" (>3-10%) *(Fig. 2, Fig. 1-f)*. Very low values of schizocytes were found, that is, another poikilocytotic form of RBC. Their presence on blood smears was "rare" (0.05-0.5%) in all study groups and their subgroups *(Fig. 2)*.

DISCUSSION

It is widely known that the contact of bioactive materials with a tissue surface leads to specific biological response. Many authors [8-10] have determined that acute inflammation is not always present. Our research did not determine any acute form of inflammation, which may be explained by the fact that lymphocytes and neutrophils, and the total number of WBC in all the experimental and control groups were within the reference ranges. Likewise, the lack of inflammation processes may be related to the appropriate application of dental treatment on the pulp, as well as the suitable subcutaneous surgical procedures.

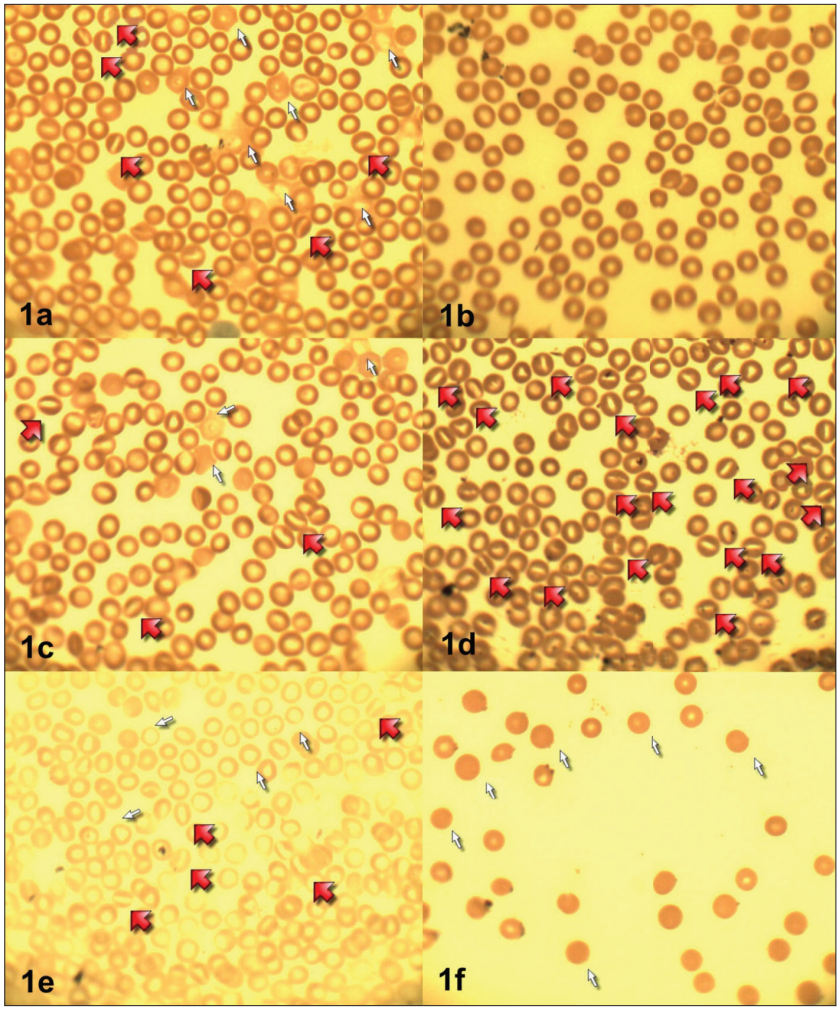

Fig 1. Poikilocytic forms of RBC, peripheral blood smear, magnification 1000x; **a-** *Red arrow* - stomatocytes, *white arrow* - RBC in the process of destruction; **b-** Healthy RBC; **c-** *Red arrow* - stomatocytes, *white arrow* - RBC in the process of destruction; **d-** *Red arrow* - stomatocytes; **e-** *White arrow* - anulocytes, *red arrow* - stomatocytes; **f-** *White arrow* - spherocytes

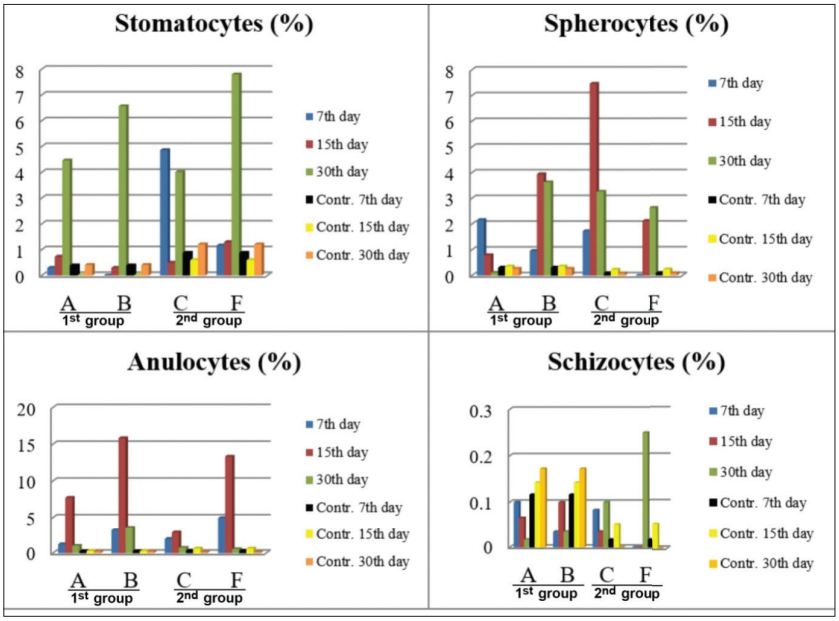

Fig 2. Percentage ratios of poikilocytic RBC forms

Rats are animals with a pronounced lymphocyte blood chart [11,13,23,24]. Our research recorded a trend of dominant percentage values of lymphocytes compared to other cells of the leukocyte type *(Table 1)*. The significant differences in the mean values of basophils, acidophils and monocytes could be explained by their low and narrow physiological benchmarks [11,23,24]. Thus, they may be ignored. Our results correspond to those in Leprince et al.[25].

The lower values of PLT and significant differences between the mean values of the control and on day 30 ($P<0.05$) could be explained by the loss of PLT caused by their role in stopping bleeding after surgery, anaesthetic application, or blood sampling due to haematological analysis.

Osan et al.[15] studied the biocompatibility of dental cements (Ivoclar D and DSM and Vitadur D and DSM) after subdermal implementation, using the bone marrow and blood corpuscles of the peripheral blood of experimental rats.

In our research, the target location of the first study group (subgroup A and subgroup B) was direct coverage of pulp. Dental pulp in rats is fairly innervated and vascularized, especially in peripheral areas, containing a odontoblastic and sub odontoblastic capillary plexus.

The study recorded lower numbers of RBC, which showed no connection with the type of implanted material, on the dental pulp (subgroup A and subgroup B). In the second study group (subgroups C and F) there were significant differences related to both types of implanted cement, between the 7th and 15th days, and the 15th and 30th days of the experiment.

The results indicate expressed oligocythemia caused by the application of calcium aluminate and calcium silicate, especially because the number of RBC in the control group was within the physiological limits. Likewise, lower values of haemoglobin indicate hypochromia [26]. The values of RBC, HGB and HCT indicate the existence of hypochromic anaemia caused by the damaging influence of calcium aluminate and calcium silicate. Since MCV was within the reference values, the anaemia present may be characterised as hypochromic anaemia of the normocytic type.

Some researchers determined the link between anaemia and the effects of aluminium *in vivo* and in vitro. Independent of the aluminium compound or application method, anaemia was confirmed in mice, rabbits and rats. Its toxic influence was confirmed in mature RBC in peripheral blood, as well as in progenitor cells in bone marrow [27,28].

Likewise, hypochromic anaemia of the normocytic type was determined in animals treated with calcium silicate (subgroup B-pulp and subgroup F-subdermal tissue). The available literature does not reveal research studies showing a similar toxic effect of calcium silicate on RBC parameters.

Healthy RBC in rats have a biconcave disk shape, as in most mammals [29]. The appearance of poikilocytotic forms of erythrocytes is not rare, and is caused by biochemical or pathophysiological changes due to the influence of toxins or physical trauma to the RBC. They may directly support the occurrence of anaemia [19,26,30] independently of etiological factors. The type of abnormally shaped RBC with a lighter central area and darker periphery that resembles a ring, is called an anulocyte. It occurs in iron deficiency or hypochromia [31].

When we evaluated the effects of aluminium compounds on mature RBC *in vivo,* the results were similar to those obtained in the study by Vitori et al. [27] and Vitori et al.[28]. Research by Vittori et al.[28] proved the direct influence of aluminium citrate or aluminium chloride on mature human RBC and erythroprogenitor precursor cells in different species after 14 days of application. Their transformation into stomathocytes and acanthocytes was detected. They proved the hypothesis using an electronic microscope, which showed a significant amount of aluminium connected to the RBC membrane [28]. This undoubtedly indicated the effect of aluminium on RBC, reshaping them into stomatocytes, which finally leads to the appearance of anaemia. Microscopic examination of peripheral blood smears revealed a smaller number of RBC in the process of destruction, which represents haemolysis, meaning that the haemoglobin is leaving the RBC stroma.

Our research did not reveal the presence of acanthocytes, as recorded in other studies [28], however, there was a smaller presence of some other poikilocytotic forms of RBC, such as schizocytes. Their presence was "rare" in our study, and associated with the following: mechanical trauma of the cell during the creation of blood smears, the influence of anticoagulants, or microscopy on the edges of the blood smear [19,29]. Significance was given to the presence of spherocytes, classified as "moderate" (>3-10%). In available literature, a higher presence of this type of cells is linked to different types of anaemia [19,29,32].

The toxic influence of calcium silicate on RBC and the appearance of anaemia has not been completely explained. Our research showed that with application on to the pulp, subdermal hypochromic anaemia of the normocytic type exists, along with modest (>3-10%) to expressed (>15%) distribution of anulocytes and stomathocytes.

The effects of calcium silicate were more pronounced compared to those of calcium aluminate. There is not enough literature dealing with the effects of calcium silicate on haematological parameters in *in vitro* or *in vivo* experiments. Thus, it would be beneficial to determine the transformation of poikilocytotic RBC after treatment with calcium silicate using electronic microscopy. This is even more significant if we take into account the wide usage of calcium silicate in human and veterinary dentistry.

The influence of the tested dental cements on haematological parameters is unfavourable, leading to the appearance of hypochromic anaemia of the normocytic type. The study detected expressed anulocytosis in the groups treated with calcium silicate, and moderate stomatocytosis in the groups treated with calcium aluminate. Likewise, moderate spherocytosis was present following

the application of both cements implemented in both tissues. The aforementioned poikilocytotic forms are the vanguard of RBC destruction, in other words haemoglobin leaves the RBC stroma. The results imply the possibility of haematological disorders after cement is implanted in humans. Further studies are needed (in humans) to clarify the possible negative effects of these materials in human dentistry.

Conflicts of Interest

The authors declare no conflict of interest.

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This article has been retracted with the author's approval due to major errors (miscalculation) detected in its content.

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Case Report

Low-field Magnetic Resonance Imaging of Changes Accompanying Slipped Capital Femoral Epiphysis in a Cat

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Abstract

A one-year-old neutered Maine Coon cat was admitted to the clinic with sudden onset of lameness in the right pelvic leg persisting for around 2 days. A clinical examination revealed lack of weight bearing on the right pelvic limb, minor bilateral atrophy of gluteal muscles and acute pain upon palpation of the right hip joint. Radiographs taken in the dorsoventral projection revealed a large radiolucent area in the proximal femoral epiphysis, surface remodeling of the femoral head and subcartilaginous sclerotization in the right pelvic limb, which were indicative of slipped capital femoral epiphysis. Radiolucent foci on the femoral head was observed in the left pelvic limb. The patient was examined in the Esaote Vet-MRI Grande scanner (0.25 T). The scans revealed complete separation of the femoral head, the presence of a hematoma and bone marrow edema in the right limb, as well as widening of the growth plate, bone marrow edema and the presence of a subcartilaginous cyst in the left limb. Resection arthroplasty of the right femoral head was performed, and the slipped femoral head was subjected to a histopathological examination. The aim of this study was to evaluate the use of low-field MRI for diagnosing slipped capital femoral epiphysis.

Keywords: *Cat, Slipped capital femoral epiphysis, Hip joint, Magnetic resonance imaging, SCFE*

Bir Kedide Kaymış Femur Başı Epifizine Bağlı Değişikliklerin Düşük-alan Manyetik Rezonans Görüntüleme İle Belirlenmesi

Öz

Bir yaşlı ve kısırlaştırılmış Maine Coon ırkı kedi, sağ arka bacakta aniden başlayan ve yaklaşık 2 gün devam eden topallık şikayeti ile kliniğe kabul edildi. Klinik muayenede sağ arka bacağa ağırlık verememe, gluteal kasların hafif bilateral atrofisi ve sağ kalça ekleminin palpasyonu sırasında akut ağrı saptandı. Dorsoventral radyografik görüntülemede proksimal femoral epifizde geniş bir radyolusent alan, femur başında yüzeysel şekil değişikliği ve sağ arka bacakta kaymış femur başı epifizinin göstergesi olan sub-kartilaginöz sklerotizasyon belirlendi. Sol arka bacak femur başında radyolusent odaklar izlendi. Hasta Esaote Vet-MRI Grande cihazı ile (0.25 T) incelendi. Taramalarda sağ bacakta femur başında tam ayrılma, hematom ve kemik iliği ödeminin yanı sıra sol bacakta büyüme plağı genişlemesi, kemik iliği ödemi ve sub-kartilaginöz kist tespit edildi. Sağ femur başına rezeksiyon artroplastisi uygulandı ve kaymış femur başı histopatolojik incelemeye tabi tutuldu. Bu çalışmanın amacı, kaymış femur başı epifizinin tanısında düşük-alan MRG kullanımını değerlendirmektir..

Anahtar sözcükler: *Kedi, Kaymış femur başı epifizi, Kalça eklemi, Manyetik rezonans görüntüleme, SCFE*

INTRODUCTION

Slipped capital femoral epiphysis (SCFE) is referred to as a spontaneous fracture of the growth plate without direct trauma [1]. In human medicine this pathology is also determined as a displacement of the epiphysis on the metaphysis through the physis [2]. This progressive disease leads to complete separation of the epiphysis as a result of repeated overloading [1]. In most patients, the etiology of the disease is unknown. In human subjects, slipped capital femoral epiphysis can be caused by biomechanical, genetic and biochemical factors (for example renal failure, endocrine problems or complications after radiotherapy). A combination of these factors weakens the growth plate and increases mechanical forces acting on the epiphysis [2-5]. McNicolas et al.[6] observed that the histological characteristics of slipped capital femoral epiphysis are similar to those noted in growth plate disorders in children, and they include disrupted chondrocyte structure, chondrocyte accumulation, growth plate thickening and surface cracking.

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According to Craig [7], other characteristic features include the formation of fibrous tissue on cartilage surface, multifocal granulation and ossification. In cats older than one year, the four main factors predisposing to slipped capital femoral epiphysis include gender, reproductive status (the disease is more prevalent in neutered males), delayed growth plate closure (which normally occurs between the age of 30 to 40 weeks) and high body weight [6] Slipped capital femoral epiphysis is most prevalent in Siamese cats and domestic short-haired cats, but it has been increasingly reported in Maine Coons in the literature [1,6,8]. Joint diseases in cats often has non-specific symptoms like: lower levels of physical activity, reluctance to jump, decreased appetite, increased thirst and inability to a comfortable resting position [9,10], but SCFE can revelated sudden onset of lameness, pain upon palpation of incorrect joint and atrophy of gluteal muscles. In the present case study, slipped capital femoral epiphysis was diagnosed in a one-year-old neutered Maine Coon male.

CASE HISTORY

A 12-month-old neutered Maine Coon male cat with a body weight of 7.6 kg was admitted to the clinic with sudden onset of lameness in the right pelvic leg persisting for around 2 days. The owners reported on the patient's aggressive behavior and vocalization, but they ruled out the possibility of traumatic injury. A clinical examination revealed lack of weight bearing on the right pelvic limb, minor bilateral atrophy of gluteal muscles and acute pain upon palpation of the right hip joint. Superficial and deep sensation was confirmed in both pelvic limbs. The patient was premedicated with medetomidine (Cepetor, ScanVet, 1 mg/mL) at 0.05 mg/kg BW and butorphanol (Torbugesic, Pfizer Trading Polska, 10 mg/mL) at 0.1 mg kg/BW, and a catheter was inserted into *vena cephalica* to provide venous access. Radiographs of the right and left hip joints were performed in dorsoventral projections. Radiographs revealed a large radiolucent area in the proximal femoral epiphysis, surface remodeling of the femoral head and subcartilaginous sclerotization in the right pelvic limb *(Fig. 1)*. Radiolucent foci in the femoral head was observed in the left pelvic limb *(Fig. 2)*.

General anesthesia was induced with propofol (Provive, Claris Lifesciences, UK, 10 mg/mL) at 2 mg kg/BW. The patient was examined in the Esaote Vet-MRI Grande low-field MRI scanner (0.25 T) in sternal recumbency with the pelvic limbs extended caudally. The hip joints were positioned centrally in a dual-phased array transmit/receive knee coil No. 2. The MRI examination was performed in the Spin Echo (SE T1) sequence in the sagittal (TR 650 ms, TE 26 ms), dorsal (TR 750 ms, TE 26 ms) and transverse (TR 3000 ms, TE 120 ms) plane, in the XBONE sequence in the dorsal plane (TR 800 ms, TE 21 ms, 28 ms, 14 ms, 21 ms), and in the FSE T2 sequence in the transverse plane (TR 3000 ms, TE 120 ms). Based on the results of the

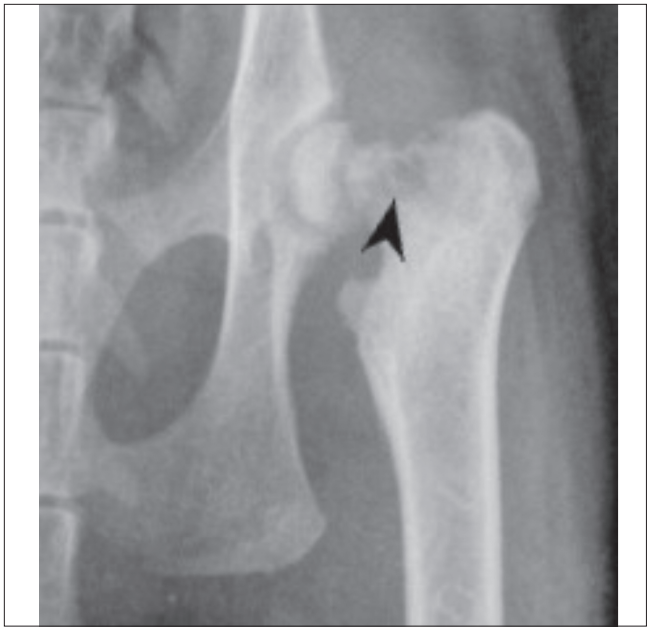

Fig 1. Dorsoventral X-ray of right hip joint in cat. Radiograph revealed a large radiolucent area in the proximal femoral epiphysis *(arrow head),* surface remodeling of the femoral head and subcartilaginous sclerotization

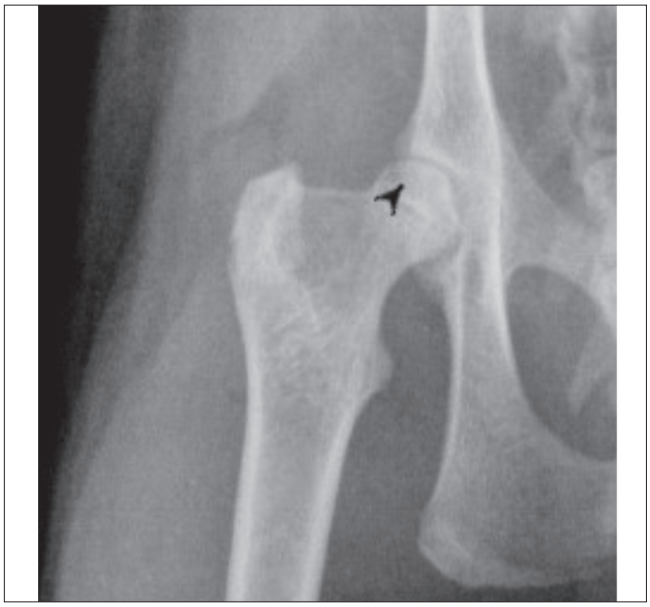

Fig 2. Dorsoventral X-ray of right hip joint in cat. The femoral head contains radiolucent foci *(arrow head)*

MRI exam, a decision was made to perform resection arthroplasty of the right femoral head. General anesthesia was maintained by inhalation of 1-2% isoflurane (Aerane, Baxter Polska, Warszawa, 100%) with fentanyl (Fentanyl WZF, Polfa, Warszawa, 50 µg/mL) administered by constant rate infusion (CRI) at 10 µg/kg/h with an infusion pump. Vital signs were monitored throughout the procedure. Arthroplasty of the right hip joint was performed in the lateral approach. The articular capsule was opened, the separated epiphysis was removed and the femoral neck was resected with a bone saw. Cephalosporin (Cefalexim,

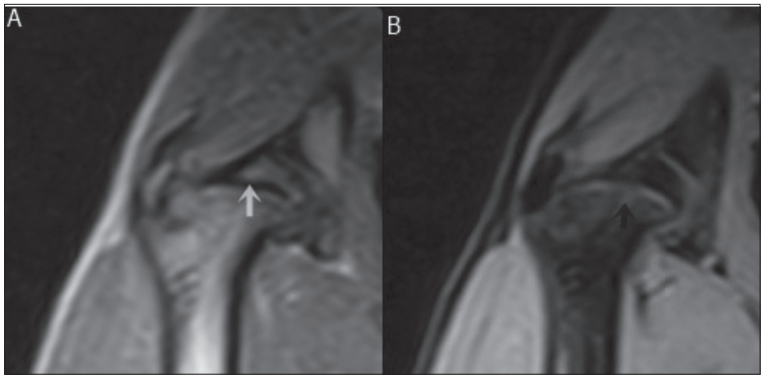

Fig 3. Dorsal plane of the left hip joint in a cat in: A) SE T1 sequence, B) XBONE sequence – water-only image. A hypointense signal in the site of growth plate widening is marked with a *white arrow.* A hyperintense signal indicative of bone marrow edema in a fat-suppressed sequence is marked with a *black arrow*

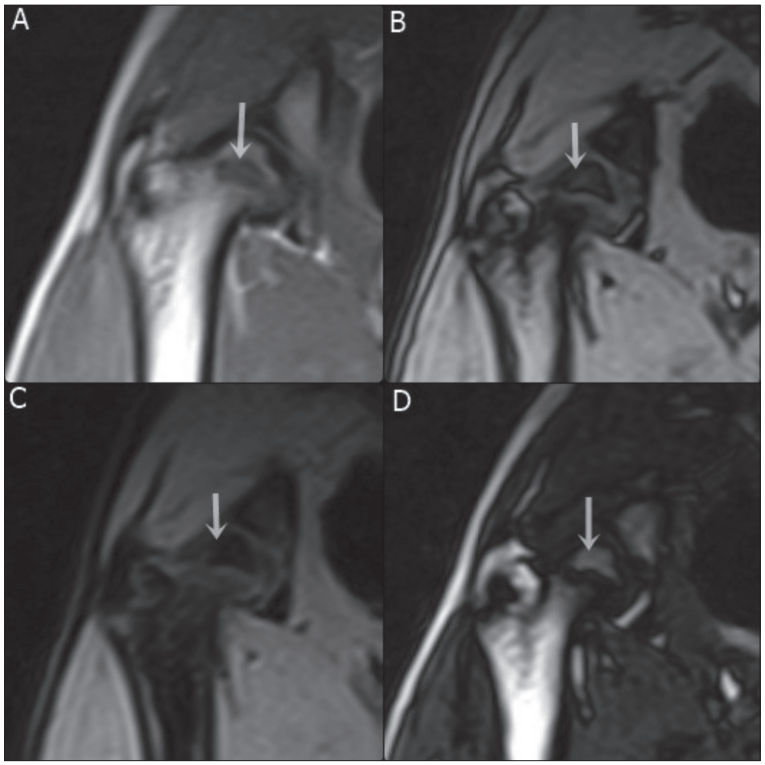

Fig 4. Dorsal plane of the left hip joint in a cat in: A) SE sequence, B) GE sequence, C) XBONE sequence - fat-only image, D) XBONE sequence - water-only image. *Arrows* indicate hypointense foci in SE and GE sequences and the fat-only image, and hyperintense foci in a fat-suppressed sequence in the femoral head which are indicative of a subchondral cyst

ScanVet Poland, Warszawa, 180 mg/1 mL) at 10 mg/kg IV and meloxicam (Metacam, Boehringer Ingelheim Vetmedica, 20 mg/mL) at 0.2 mg/kg SC were administered preoperatively, and injections were continued for 4 days. The resected femoral head was placed in 10% formalin solution and subjected to a histopathological analysis. The histopathological examination revealed uneven surface of the epiphyseal plate with multifocal cartilage lesions, proliferation of fibrous connective tissue and bone tissue, and cartilaginous metaplasia with the accumulation of chondroblasts, osteoblasts and osteoclasts. The owner has been informed about necessity of X-ray control of the left hind limb after one month, but he disagreed for any further treatment, due to non-visible symptoms of lameness in left limb.

The MRI examination of hip joints revealed pathological changes in the right and left limb. T1 hypointense widening of the growth plate and a hyperintense signal in the XBONE sequence in the "water-only" image which is characteristic of bone marrow edema were noted in the left limb *(Fig. 3)*. An oval-shaped change measuring 8 mm x 4 mm x 3 mm in the left femoral neck produced a hypointense signal in SE T1 and XBONE sequences (in GE and "fat-only" images) *(Fig. 4)*. A hyperintense signal in the region of the described change was obtained in the "water-only" image in XBONE and FSE T2 sequences. The characteristic of signal changes suggested the presence of subchondral cysts. In the right limb, the femoral head was clearly separated from the neck, and a hematoma was detected between the separated fragments based on a signal with varied intensity and foci of high signal intensity in fat-suppressed SE T1 and FSE 2 sequences and a hypointense signal in a water-suppressed sequence *(Fig. 5)*. Minor hypointense foci indicative of fibrous tissue proliferation was observed in the region of the separated femoral head in SE T1 and FSE T2 sequences. A hyperintense signal characteristic of bone marrow edema was also noted in the "water-only" image in the XBONE sequence.

DISCUSSION

Magnetic resonance imaging is not a specific test for diagnosing slipped capital femoral epiphysis, but it is helpful in evaluating the accompanying complications such as chondrolysis or osteonecrosis [11]. There is only one published report on the use of MRI for diagnosing slipped capital femoral epiphysis in cats, but the obtained images were not described [1]. The changes that accompany non-traumatic separation of capital femoral epiphysis have also been studied in pigs and dogs [12] In the MRI exam, the above changes were identified as minor hypointense foci in the femoral head in SE T1 and FSE T2 sequences. The femoral head was completely separated in the right limb. In the discussed case, a hematoma was detected between the slipped capital femoral epiphysis and the femoral neck. The MRI signal of a hematoma is determined by the breakdown products of hemoglobin [13], and it varies in different stages of hematoma organization. Initially, high oxyhemoglobin content generates a high-intensity signal in fat-suppressed T2-weighted sequences and a low-intensity signal in T1-weighted sequences. Deoxyhemoglobin is produced

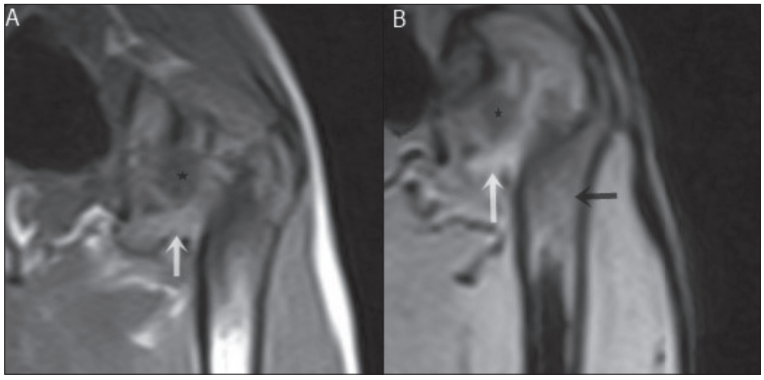

Fig 5. Dorsal plane of the right hip joint in a cat in: A) SE T1 sequence, B) XBONE sequence - water-only image. A hyperintense signal in the site of the hematoma is marked with a *white arrow.* A hyperintense signal indicative of bone marrow edema in a fat-suppressed sequence is marked with a *black arrow.* A hypointense signal indicating the loss of cartilaginous tissue and proliferation of fibrous tissue in the femoral head is marked with an *asterisk*

approximately one h after hematoma formation, and it produces a hypointense signal in T2-weighted images. After 24 h, intracellular methemoglobin appears as a hyperintense mass in T1-weighted images. Erythrocytes are decomposed one week after hematoma formation, and they release methemoglobin which increases signal intensity in T1- and T2-weighted images. In the last stage, hemosiderin-laden macrophages accumulate at the periphery of the hematoma and decrease signal intensity in all sequences [14]. A varied signal with hyperintense foci generated by methemoglobin in SE T1 and FSE T2 sequences is indicative of hematoma organization, and it suggests that the hematoma was formed more than 24 h ago. On the radiograph of the right limb the femoral head was clearly separated from the neck and MRI was performed for confirming the diagnosis. The hematoma formation and bone marrow edema were not visible on the X-ray.

Growth plate widening and a regular area on the femoral neck with a signal characteristic of fluid components were noted in the left limb. Oval or round areas characterized by low-intensity signals in T1-weighted images and high-intensity signals in T2-weighted images can suggest the presence of subchondral cysts [15]. In the absence of complete separation and dislocation of the femoral head, the above changes could be indicative of the onset of slipped capital femoral epiphysis [11,16]. The changes in the growth plate of the left leg observed in MRI scans were not visualized in radiographs. MRI detected early physeal changes of onset slipped capital femoral epiphysis when X-rays were normal. MRI can revelated early bone marrow edema and growth plate widening, which can be not visible on X-ray. Magnetic resonance imaging is a useful tool for detecting SCFE in contralateral asymptomatic hip.

MRI scans supported the visualization of pathological changes in both pelvic limbs of the evaluated cat. Growth plate widening, bone marrow edema and the presence of a subcartilaginous cyst were indicative of the onset of slipped capital femoral epiphysis in the left limb. Complete separation of the femoral head, the presence of a hematoma and bone marrow edema in the right limb were indicative of advanced progression of the disease in the right limb. MRI scans supported the identification of changes that were not visualized in radiographs, which facilitated prognosis and the choice of the appropriate treatment.

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Case Report

Abdominal Wall Hibernoma in a Cat: A Case Report [1]

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[1] *This case report was presented as a poster presentation at the 14th International Continuing Education Congress Turkish Small Animal Veterinary Association (8-10.11.2019)*

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Abstract

Hibernoma is a very rare benign tumor of brown fat tissue which is found in hibernating and non-hibernating mammals. Until now, it has been reported in rats, dogs, and human beings. In this case report, a thirteen-year-old, spayed, female Siamese cat was examined for intestinal motility disorder. Local obstruction of the colon descendens was observed on radiographs 72 h after contrast medium administration. In order for the abdominal organs to be examined, diagnostic laparotomy was performed. A mass 1 cm in diameter located on the abdominal wall showing adhesion to mesenterium of the colon descendens was observed and completely removed. Post-operative treatment was maintained by intestinal diet, metoclopramide, and enrofloxacin administration. Histopathological examination of the mass revealed hibernoma, a benign tumor of brown fat tissue. Further immunohistochemical analyses were performed to evaluate the origin and behavior of the tumor by using S-100 and osteopontin antibodies. As a result, immunohistochemical staining was positive for S-100 and was weakly positive for osteopontin antibodies. In the presented report, a case of hibernoma in a cat was described with the clinical, histopathological, immunohistochemical findings and treatment procedure.

Keywords: *Abdominal wall, Brown fat tissue, Cat, Hibernoma, Tumor*

Bir Kedide Abdominal Hibernoma: Olgu Sunumu

Öz

Hibernoma, kış uykusunda ve kış uykusunda olmayan memelilerde bulunan, çok nadir görülen, iyi huylu bir kahverengi yağ dokusu tümörüdür. Bugüne kadar farelerde, köpeklerde ve insanlarda bildiren raporlar bulunmaktadır. Bu olgu sunumunda; on üç yaşında, kısır, dişi bir Siyam kedisi bağırsak motilite bozukluğuna yönelik muayene edildi. Kontrast madde verilmesinden 72 saat sonra radyografilerde colon descendens'te lokal obstruksiyon gözlendi. Abdominal organların incelenmesi için diyagnostik laparotomi yapıldı. Karın duvarında, colon descendens ile mezenteryum adezyonuyla karakterize 1 cm çapında bir kitle gözlendi ve kitle tamamen çıkarıldı. Ameliyat sonrası tedavi; intestinal diyet, metoklopramid ve enrofloksasin uygulamasıyla sürdürüldü. Kitlenin histopatolojik incelemesinde; kitleye kahverengi yağ dokusunun iyi huylu tümörü olan Hibernoma tanısı konuldu. Tümörün orijinini ve biyolojik davranışını değerlendirebilmek için S-100 ve osteopontin antikorları kullanılarak, immünohistokimyasal analizler yapıldı. Sonuç olarak, immünohistokimyasal boyamalarda tümör dokusu S-100 için kuvvetli pozitifken, osteopontin'e karşı çok zayıf pozitif yanıt verdi. Bu yazıda, bir kedide saptadığımız hibernoma klinik, histopatolojik, immünohistokimyasal bulgular ve tedavi prosedürü birlikte sunulmuştur.

Anahtar sözcükler: *Hibernoma, Karın duvarı, Kahverengi yağ dokusu, Kedi, Tümör*

INTRODUCTION

Brown fat was defined by Velch in 1670 as a specialized fat tissue found in hibernating and non-hibernating animals; such as rats, monkeys, cats, rabbits, and human beings [1]. Brown fat tissue allows the non-shivering thermoregulatory function by the dissipation of energy through the production of heat in the condition of cold induced stress. Therefore, brown fat tissue plays a particular role for small mammals, neonatal human beings, rodents and hibernating animals [2-4]. Hibernoma is rare, benign soft tissue tumor of this specialized brown fat and can occur both in human beings and animals. It was first described as "pseudolipoma" in human beings by Merkel in 1906 [1]. Thereafter, the term so-called "hibernoma" was given by Gery in 1914 [1], because of its morphological resemblance

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to the hibernating gland in hibernating animals. This case report presents clinical, histopathological and immunohistochemical findings of a hibernoma in a cat.

CASE HISTORY

A thirteen-year-old, spayed, female Siamese cat with intestinal motility disorder, including difficulties in defecation and constipation, was brought to the veterinary clinic. On physical examination, the cat was found to be severely lethargic, dehydrated and the body condition score was 6/9. In addition to these findings, the cat also had chronic periodontal disease. Body temperature and respiratory examination were normal. Laboratory findings, total blood test and biochemistry parameters indicated no signs for anemia. In biochemistry total protein (8.3 g/dL), albumin (4.0 g/dL), glucose (158 mg/dL), blood urea-nitrogen (43.0 mg/dL) and globulin (4.3 g/dL) counts were found mildly high that was found to be associated with severe dehydration. The increased level of the globulin was found related to the periodontal disease. Severe abdominal defense was observed during abdominal palpation. On ultrasonographical examination, the peristalsis of the intestine was found abnormal and distention was observed in the area of the large intestine. Severe intestinal distention was observed at the radiography, and the patient was examined by further radiological tests with contrast material to differentiate obstructive or non-obstructive distention. As a result, local obstruction of the colon descendens was observed on radiographs 72 h after iohexol contrast media (Omnipaque; GE Healthcare) administration (700 mg iodine/kg with an iodine concentration of 300 mg iodine/mL diluted with tap water until a total volume of 10 mL/kg, via an orogastric tube) *(Fig. 1)*. The patient then was referred to median laparotomy to determine the reason of the obstruction. Surgical laparotomy was performed under general anesthesia and a mass 1 cm in diameter was found on the abdominal wall showing adhesion with increased adipose tissue to mesenteric margin of the colon descendens. The mass and the increased adhesive adipose tissue were completely removed, and the omentalization of the area was carried out. The mass was submitted to Pathology Department of IUC Veterinary Faculty for histopathological examination. Postoperative treatment was provided with intestinal diet (digestive care i/d, Hills) and with metoclopramide (Metpamid; Sifar) 1.0 - 2.0 mg/kg/d IV as a constant rate infusion with isotonic serum for 72 h as a parenteral fluid replacement to treat the severe dehydration and general condition of the animal. Then the additional application for metoclopramide (Metpamid; Sifar) was initially provided with 0.2 mg/kg, SC, q8h for 4 days and then given orally

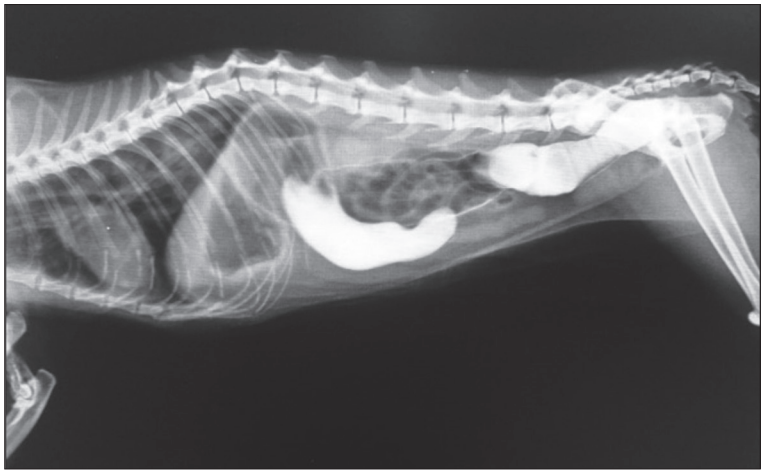

Fig 1. Radiographic appearance of the cat 72 h after contrast agent administration. The obstruction could be seen in the colon descendens

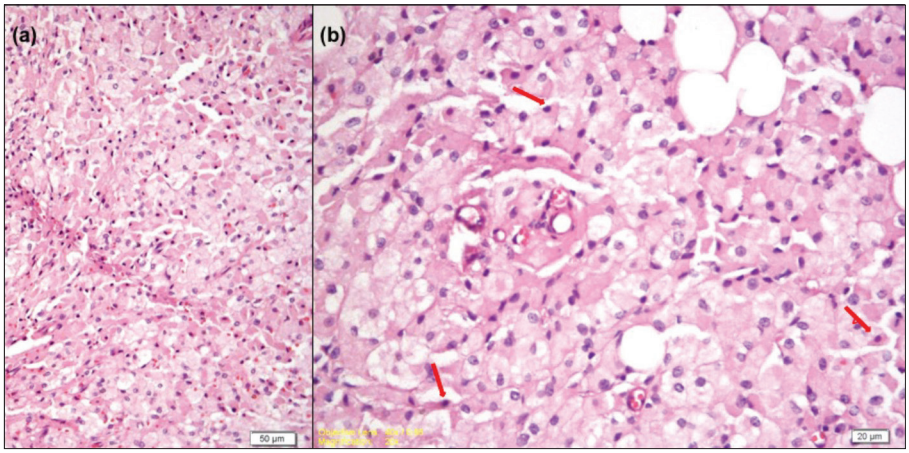

Fig 2. a,b: Abdominal mass. The cells have eosinophilic cytoplasm with numerous small vacuoles and centrally or eccentrically located nucleus (some of these cells have picnotic nucleus, red arrows), Hematoxylin and eosin (X 200 and X 400)

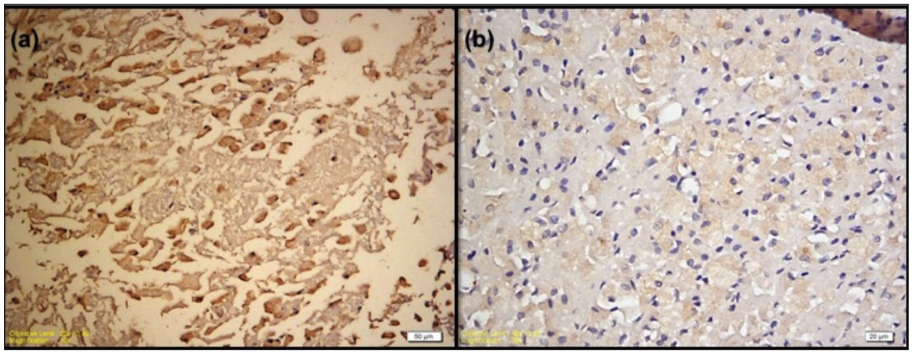

Fig 3. a: Abdominal mass. Brown fat tissue cells show moderate to a strong positive reaction in their cytoplasm after staining with anti-S100 antibody, Peroxidase immunohistochemistry (X 400); **b:** Abdominal mass. Brown fat tissue cells give weak positive immune reaction to osteopontin, Peroxidase immunohistochemistry (X 400)

for 7 days, and with enrofloxacin (Baytril; Bayer) 5 mg/kg, q24h, SC, for 10 days.

The mass was cut into small pieces, then was fixed in 10% formaldehyde solution. The tissue pieces were routinely processed and embedded in paraffin blocks. Tissue sections of 4 µm thickness were cut and stained with Hematoxylin & Eosin for histological examination. In addition, immunohistochemical analyses were performed on tissue sections using S-100 protein and osteopontin antibodies. The tissue sections were incubated in citrate buffer (pH.6) in the microwave oven at 800W for 20 minutes for the antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Tissue sections were covered with anti S-100 protein antibody (RTU-S100p, Novocastra, Leica, Newcastle, UK) (ready to use), and anti-osteopontin antibody (Ab8448, Abcam, Cambridge, UK) (ready to use) for 1 h at room temperature. Thereafter, incubation with peroxidase-conjugated HRP-conjugated secondary antibodies (ready to use) (859043, Life Technologies, Frederick, USA) were applied on the sections for both primary antibodies. The immunochemical reaction was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) (002020, Invitrogen, Frederick, USA).

On macroscopic examination, the mass was 1 cm in diameter, well demarcated, greasy, lobulated, grey to light brown color and had soft consistency. The cut surface of the mass was also light brown color and in soft consistency. Histopathological examination revealed randomly distributed lobules, which were supported by thin fibrovascular septa. The cells were large, round to polygonal adipocytic cells and ramified small capillaries. They had eosinophilic cytoplasm with numerous small vacuoles and centrally or eccentrically located nucleus *(Fig. 2-a,b)*. In addition, there were only a few mononuclear cells scattered between the brown fat tissue cells. According to these typical histological properties, the case was diagnosed as hibernoma. Immunohistochemistry for S-100 revealed a moderate to strong positive reaction in the cytoplasm of the brown fat tissue cells *(Fig. 3-a)*, but weak positive immune reaction to osteopontin was observed in the cytoplasm of the brown fat tissue cells *(Fig. 3-b)*.

DISCUSSION

Hibernoma is an uncommon, benign soft tissue tumor, and so far has been reported in human beings [5-7], rats [8,9] and dogs [10-13]. Hibernoma is thought to have derived from residual brown fat tissues which are most frequently found in the periscapular and interscapular region, the neck, axilla and shoulder, thorax, retroperitoneum and less frequently in thigh, popliteal fossa, buttock, intracranial sites [1] and breast [14]. Among them, thigh has been reported as the most common anatomical localization for hibernomas in human medicine [5,15], rather than neck, shoulder, back, axilla, mediastinum and abdominal cavity [5,16]. In veterinary medicine, brown fat tissue has been demonstrated in interscapular, perirenal, and caudal subcutaneous adipose depots of adult cats [17]. On the other hand, hibernomas have been documented in varying locations in rats [8] and in dogs, they have been documented in corneum [12], subcorneum [13] and omentum [10,11]. In this case report, hibernoma was diagnosed in the abdominal wall and showed adhesion to the mesenteric margin of the colon descendens.

Clinical observation of hibernoma manifests slowly growing-painless soft tissue mass [1]. However, the clinical manifestation in this case was different. The adhesion of the mass and increased adipose tissue together had a compressive effect on the serosa of the colon which led to intestinal motility disorder.

Macroscopic findings of the tumor in this case was compatible with the macroscopic description for hibernoma [1]. Histo-pathology revealed typical features of hibernomas by showing large, oval to polygonal brown fat cells with small capillary proliferation and the stromal background. The adipocytes were multivacuolated and had abundant, eosinophilic cytoplasm and a small central nucleus. The histopathologic features were found consistent with the pathological findings of hibernoma described in a study [5]. In the same study, histopathological findings of hibernoma have been divided in different variants, which are myxoid, spindle cell and lipoma-like hibernomas. Histopathologic findings in this case were attributed to lipoma-like hibernoma among these variants.

In this case, the positive immunoreactivity with S-100 protein supported the findings of other studies in which hibernomas give positive reaction to S-100 protein [5,10,18] in 85% of all hibernoma cases and in all histopathologic variants [5]; although it is not a specific marker for hibernoma. Furthermore, the case was also evaluated by osteopontin immunostaining to evaluate the biological behavior of the tumor. Because osteopontin is an important molecular marker for determining the behavior of the soft tissue tumors and extensive studies confirm that highly expressed osteopontin refers a malignant potential [19]. Here, a very weak positive reaction obtained with osteopontin was attributed to be the benign behavior of hibernoma.

Curative treatment can be possible by complete surgical excision for hibernomas [5,20,21]. Reoccurrence does not happen and neither metastases nor malignancy has been reported [1]. In this report, the intestinal motility disorder of the cat was treated by the complete removal of the mass and omentalization of the surgical area. In addition, post-operative treatment was provided to treat the general condition of the cat. No recurrence has been observed since the post-operative period.

As a conclusion, this case report contributes to literature by presenting hibernoma in a cat. Histopathologic features of the tumor were found characteristic for hibernoma. Immunohistochemical findings for S-100 were compatible with the previous studies. Furthermore, the weak immune reaction to osteopontin confirmed the benign behavior of hibernoma. The intestinal motility disorder of the cat was improved by the surgical excision of the mass. The condition of the cat is known to have remained stable since the termination of the treatment.

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Letter to the Editor

Two Cases of "Celox" Gauze Application to Control Bleeding from the Canine Popliteal Artery

(İki Köpekte Popliteal Arter Kanamasının Kontrolü İçin "Celox" Gazlı Bez Uygulaması)

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Dear Editor,

Severe arterial bleeding caused by trauma is potentially life-threatening and requires immediate surgical attention. Many life-saving procedures in veterinary practice are based on the solutions that have been developed in human medicine. One of such procedures relies on hemostatic dressings to manage profuse bleeding from large vessels [1,2]. Hemostatic dressings form a micro-adhesive physical barrier by absorbing water from the blood, activating platelets and increasing clotting capabilities at the site of injury. Celox Gauze contains chitosan granules which effectively stop bleeding from arteries. Chitosan works independently of the physiological clotting mechanism and produces a hemostatic plug by binding to red blood cells and platelets [3-5]. This lettering describes the use of the Celox Gauze hemostatic dressing for controlling bleeding from an injured popliteal artery in two dogs.

Celox gauze was applied in two dogs: a male Rottweiler aged 5 years with body weight of 46 kg and a male German Shepherd aged 6 years with body weight of 39 kg. Both patients had undergone surgical treatment of anterior cruciate ligament injury. In both cases, the popliteal artery was damaged when the surgical band was passed behind the lateral condyle of the femur with the use of a sharp surgical needle during the stabilisation of the knee joint. Immediately after injury, the wound was compressed with sterile tampons on the lateral side of the popliteal fossa. The tampons were removed, and the Celox Gauze hemostatic dressing (MedTrade Products Ltd., Crewe, UK) was applied approximately 3 min later. Celox Gauze was tightly packed in the wound cavity, covered with sterile tampons and manually compressed. The compression time was approximately 20 min. The tampons were observed for around 2 min to monitor signs of bleeding. None of the tampons absorbed blood. Celox Gauze was carefully removed, and the wound was observed for approximately 3 min. Hemostasis was effectively achieved in both cases. Surgical repair of anterior cruciate ligament injury was continued. The injured popliteal artery was not sutured. Soft tissues in the surgical site were tightly sutured. Both dogs were intravenously administered Sterofundin (Braun, Melungen, Germany) at 5 mL/kg/h throughout surgery, and the dose was increased to 10 mL/kg/h during haemorrhage control. The temperature of the operated limb was monitored for two days after the procedure.

Both dogs achieved normal limb loading 6 weeks after surgery, and the owners were satisfied with the outcome. The temperature of the operated limb was normal during examinations performed 2 and 4 weeks after the procedure. Infections of the skin or subcutaneous tissues were not observed in the surgical site. The area of the wound was not swollen or bruised. One year after surgery, the Rottweiler was again admitted to the clinic for surgical treatment of anterior cruciate ligament injury in the other limb.

There is a general scarcity of published research into the

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use of hemostatic dressings in dogs. A hemostatic dressing containing chitosan acetate was successfully applied to control bleeding from the canine femoral artery [6]. Chitosan-based hemostatic dressings were also effectively used in rats [5,7]. The application of hemostatic dressings in pigs was more extensively researched [8-10]. Kozen et al.[4] reported that Celox Gauze was as effective as two other hemostatic dressings, and it significantly improved survival rates in comparison with standard dressings. In contrast, in the different study observed no differences in the effectiveness of standard gauze dressings and other hemostatic dressings applied to small wounds that were caused by penetrating injuries in sites that were difficult to compress [11]. In both described cases, hemostasis was effectively achieved with Celox Gauze. The evaluated dressing was applied to the wound for 20 min without causing soft tissue irritation in the wound area. After an injury to the popliteal artery, blood was supplied to the thigh mainly by the deep femoral artery.

As a result, in this lettering, the clinical outcomes in the presented cases indicate that the Celox Gauze hemostatic dressing effectively controls bleeding from the canine popliteal artery.

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