ATATURK
UNIVERSITY
PUBLICATIONS

Veterinary Sciences *and* Practices

Formerly: *Atatürk University Journal of Veterinary Sciences*
Official journal of Atatürk University Veterinary Sciences

Volume 20 • Issue 2 • August 2025

EISSN 2822-3608
https://dergipark.org.tr/en/pub/vetsci

Veterinary Sciences *and* Practices

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Veterinary Sciences *and* Practices

ABOUT

Veterinary Sciences and Practices is a peer-reviewed, open access, online-only journal published by Atatürk University.

Veterinary Sciences and Practices is published triannually in Turkish and English, with issues released in April, August, and December. Beginning on October 17, 2023, Veterinary Sciences and Practices will exclusively consider articles in English. However, Turkish abstracts will still be included in English articles alongside the English abstracts. A language editor will be responsible for translating the English abstracts of accepted papers into Turkish.

Journal History
As of 2022, the journal has changed its title to Veterinary Sciences and Practices.

Current Title
Veterinary Sciences and Practices
EISSN: 2822-3608

Previous Title (2006-2021)
Atatürk Üniversitesi Veteriner Bilimleri Dergisi
ISSN: 1306-6137
EISSN: 2147-9615

Veterinary Sciences and Practices currently indexed in Scopus, DOAJ, EBSCO, EMBASE, CABI, CAB Abstract, CAS, China National Knowledge Infrastructure (CNKI), Gale Cengage, and TUBITAK ULAKBIM TR Index.

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The target audience of the journal includes specialists and professionals working and interested in all disciplines of veterinary medicine.

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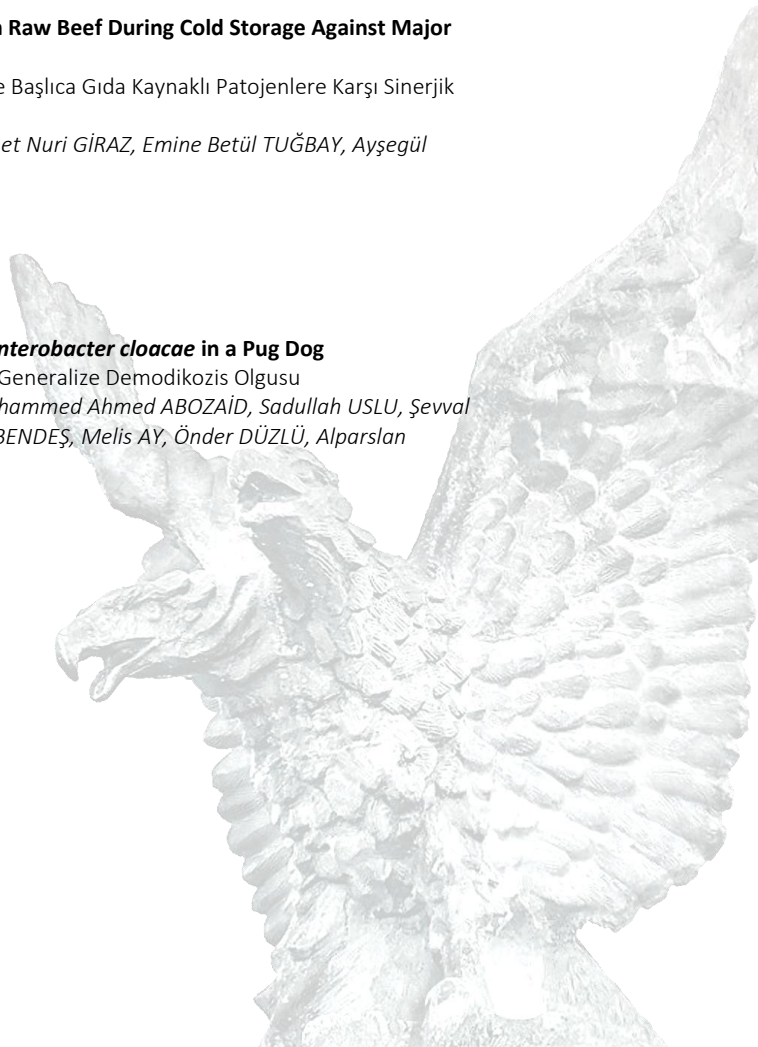

ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1646232

Investigation of Extended-Spectrum Beta-Lactamase Producing *Escherichia Coli* in Milk on Dairy Cattle Farm

Çiftliklerden Alınan İnek Sütlerinde Genişlemiş Spektrumlu Beta-Laktamaz Üreten *Escherichia Coli*'nin Araştırılması

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ABSTRACT

The present study was conducted to investigate the presence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) in cattle farm milk mixtures. Thirty two farm milks from 32 farms in Gölhisar district of Burdur city (Türkiye) were concurrently cultured on MacConkey agar plates supplemented with either 2 µg/mL cefotaxime or 2 µg/mL ceftazidime. After incubation at 37°C for 24 h under aerobic conditions, biochemical/physiological tests were applied to the lactose positive colonies for identification of *E. coli* in the cultures. Subsequently, determination of ESBL production in *E. coli* isolate was done by the combined disk method. In order to determine the ESBL class, the isolates were screened for CTX-M, SHV and TEM genes by polymerase chain reaction (PCR). After the selective culture of the milk samples, one (1/32, 3.12%) E. coli was isolated. The isolate confirmed as an ESBL procedure by the combined disc method harbored the CTX-M group 1 ESBL gene, but tested negative for TEM and SHV genes. This study showed that ESBL-producing *E. coli* could be found at low level in farm milks of the cattle farms in the region. Although the source of the ESBL-producing *E. coli* isolated from the farm milk mixture was not identified in this study, it is important that this bacterium was detected in the milk to be consumed

Keywords: Beta-lactamase, cattle milk, CTX-M gene, extended-spectrum beta-lactamase, *Escherichia coli*

ÖZ

Bu çalışma, sığır çiftliği süt karışımlarında genişlemiş spektrumlu beta-laktamaz (ESBL) üreten Escherichia coli'nin varlığını araştırmak amacıyla yapıldı. Burdur ili Gölhisar ilçesindeki 32 çiftlikten alınan 32 çiftlik sütü, 2 µg/ml sefotaksim veya 2 µg/ml seftazidim ilave edilmiş MacConkey agar besi yerlerine eş zamanlı olarak ekildi. Aerobik şartlarda 37°C'de 24 saat inkübasyonun ardından, kültürlerde *E. coli*'nin tanımlanması amacıyla laktoz pozitif kolonilere biyokimyasal/fizyolojik testler uygulandı. Daha sonra *E. coli* izolatında ESBL üretiminin tespiti kombine disk yöntemiyle yapıldı. ESBL sınıfını belirlemek amacıyla, izolatlarda CTX-M, SHV ve TEM genleri polimeraz zincir reaksiyonu (PZR) yöntemiyle tarandı. Süt örneklerinden selektif kültür yapıldıktan sonra 1 adet (1/32, %3,12) *E. coli* izole edildi. Kombine disk yöntemiyle ESBL ürettiği doğrulanan izolatın CTX-M grup 1 sınıf ESBL genini taşıdığı, TEM ve SHV sınıflarına sahip olmadığı belirlendi. Bu çalışma, bölgedeki sığır işletmelerinin çiftlik sütlerinde ESBL üreten *E. coli*'nin düşük düzeyde bulunabileceğini göstermiştir. Bu çalışmada bir çiftlikten elde edilen çiftlik sütü karışımında tespit edilen ESBL üreten *E. coli* izolatının kaynağı araştırılmamış olsa da, tüketilecek sütte bu bakterinin tespit edilmiş olması önemlidir.

Anahtar Kelimeler: Beta-laktamaz, CTX-M geni, *Escherichia coli*, genişlemiş spektrumlu beta-laktamaz, sığır sütü

Received/Geliş Tarihi: 24.02.2025
Accepted/Kabul Tarihi: 26.05.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Gücüöz SK, Pehlivanoğlu F. Investigation of extended-spectrum beta-lactamase producing *Escherichia coli* in milk on dairy cattle farm. *Vet Sci Pract*. 2025;20(2):57-63.

Atıf: Gücüöz SK, Pehlivanoğlu F. Çiftliklerden alınan inek sütlerinde genişlemiş spektrumlu beta-laktamaz üreten *Escherichia coli*'nin araştırılması. *Vet Sci Pract*. 2025;20(2):57-63.

INTRODUCTION

The emergence of multidrug resistance in Gram-negative bacteria of animal origin, driven by antibiotic use in livestock, poses a significant threat not only to animal health but also to human health and ecological balance. The resistance developed by gram-negative bacteria by producing beta-lactamase enzymes, which destroy beta-lactam class antibiotics in particular, is very critical because such resistance is genetically mediated and the beta-lactamase genes can be horizontally transferred among bacteria. In addition, the high diversity of beta-lactamase enzymes makes the situation even worse because the substrate (antibiotic) specificity of each beta-lactamase variety varies.[1] Extended-spectrum beta-lactamases (ESBL), which constitute an important group in this diversity, are produced especially by bacteria belonging to the *Enterobacterales* family and have increased in both microflora and pathogenic strains in recent years.[2,3]

ESBL production by bacteria makes them resistant to 1st-4th generation cephalosporins, penicillins and monobactams.[1] In recent years, the increased use of 3rd and 4th generation cephalosporins in farm animals is thought to play an additional role in the increase of ESBL-producing Gram negatives.[4]

There are multiple classes of ESBL genes identified in gram-negative bacteria. The most common of these in *E. coli* isolates are TEM (temoneira), SHV (sulfhydryl variable genes) and CTX-M (cefotaximase) and the isolation proportion of *E. coli* isolates, especially those carrying variants in the CTX-M class, has increased in the last 20 years.[5] This is the case for *E. coli* strains isolated from both farm animals and humans.[6]

The presence of ESBL-producing *E. coli* has been demonstrated in cattle populations in different parts of Türkiye in the last 15 years.[7-10] Although there are some studies in Türkiye on the presence of ESBL-producing *E. coli* in milk obtained from farms, there are not sufficient numbers.[11-14] In terms of cattle farm milks of Burdur city of Türkiye, there is no research conducted or data available on the presence of ESBL-producing *E. coli* and/or other Gram negatives. Given that approximately two-thirds of the population in Burdur is engaged in agriculture and animal husbandary, understanding the microbiological quality of dairy products is particularly relevant for regional public health. Burdur, with 40% of its economy based on milk production, has an important share in this field with over a thousand tons of raw milk production per day. Burdur livestock sector has reached 195 thousand cattle and 365 thousand sheep by 2023.[15] Gölhisar district has an important place in Burdur livestock and the cattle population has reached 15,500 according to 2022 data. The area of Gölhisar district of Burdur city is 575.820 km².[16]

The presence of ESBL-producing *E. coli* in farm milks may be due to various reasons. One of these may be subclinical mastitis caused by ESBL-producing *E. coli*. Others may be fecal-environmental contamination due to poor milking hygiene, cross contamination due to poor cleaning and disinfection of milk tanks and post-milking contamination of bacteria from humans into milk. For whatever reason, the presence of ESBL-producing *E. coli* in milk to be consumed is not a desirable situation and should be evaluated from One-health perspective.

By this study, we aimed to investigate the presence of ESBL-producing *E. coli* in farm milk mixtures brought to a milk collection center which is in Gölhisar district of Burdur city (Türkiye) and to determine the class of ESBL genes in the isolate(s) to be determined.

MATERIALS AND METHODS

This study was approved by the Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Date: 02.10.2024; Decision Number:1373).

Samples

In our study sampling unit consisted of farm milk which is mixture of milk from the cows on a farm. A visit was conducted to a milk collection unit which is in Golhisar district of Burdur province where 32 farm milk samples were collected from 32 dairy cattle farms located in Gölhisar district. At least 5 milliliters of milk were taken into sterile tubes from the containers of farmers and the milk samples were transferred to the laboratory under cold conditions within 2 hours at most.

Selective Isolation and Identification

Milk samples were simultaneously plated on MacConkey agar (MCA) medium (Merck) supplemented with 2 µg/ml ceftazidime (Sigma-Aldrich) or 2 µg/ml cefotaxime (Sigma-Aldrich). After the cultures were incubated at 37°C for 24 h under aerobic conditions, the lactose-positive colonies were passaged onto Tryptic Soy agar (TSA) medium (Merck) to obtain pure culture. Then, A Gram negative rod with positive lactose and glucose utilization and no H_2S production in TSIA test tube, positive mannitol utilization, positive indole test, negative urease test, positive catalase test, negative citrate test, negative oxidase test and positive nitrate reductation test results was accepted as *E. coli*.[17]

ESBL Confirmation Test

Subsequently, ESBL production of the isolates was determined by the combined disc method, following the Clinical and Laboratory Standards Institute Standards (CLSI).[18] First, a few colonies were taken from the 24-hour culture of the isolate on TSA medium, its suspension was prepared in Tryptic Soy broth (TSB)(Merck) and turbidity was adjusted to 0.5 McFarland standard. After this suspension is spread on Mueller Hinton Agar (MHA) medium (Merck), cefotaxime (30 µg), cefotaxime-clavulanic acid (30/10 µg), ceftazidime (30 µg) and ceftazidime-clavulanic acid (30/10 µg) discs (Bioanalyse, Türkiye) with a distance of at least 3 cm between them were placed. Then, the petri dish was incubated at 37°C for 24 h and the inhibition zone diameters around the discs were measured. The inhibition zone around at least one of the discs containing clavulanic acid was at least 5 mm wider than the inhibition zone around the disc without clavulanic acid was accepted as evidence for ESBL production. In the combined disc method, *K. pneumoniae* ATCC 700603 was used as the positive control strain producing ESBL, and *E. coli* ATCC 25922 strain was used as ESBL nonproducer for negative control.[18] In the evaluation of zone diameters, values recommended by CLSI as ≤27 mm for cefotaxime and ≤22 mm for ceftazidime were accepted as evidence of ESBL production.[18]

DNA Extraction

A suspension at McFarland 5.0 turbidity was prepared by taking colonies from 24 h culture of the isolate on TSA medium. After the suspension was frozen at -20°C for 24 h, it was kept in the heating block set at 100°C for 10 min. Then, the suspension was cooled on ice, it was centrifuged at 14.000 rpm for 5 min and finally 20 µl of the supernatant from upper phase was collected, taking care not to shake the tube, and stored at -20°C to use as template DNA in PCR tests.

PCR for Identification of Esbl Genes

Information about the primers used in PCR tests performed for the detection of CTX-M, SHV and TEM class ESBL genes is presented in Table 1.[19-23] As the control strains in PCR; DNA of *E. coli* NCTC 13461 strain was used in both CTX-M gene and CTX-M group 1 PCR, DNA of *K. pneumoniae* ATCC 700603 strain was used for SHV gene PCR, and DNA of *E. coli* ATCC 35218 strain was used for TEM gene PCR. DNA of *E. coli* ATCC 25922 strain was used as negative control for all PCRs.

Table 1. Details of the primers used in PCR tests performed for ESBL genes

Target gene	Nucleotide sequence of primer	Amplicon	Reference
CTX-M universal	F: 5'-SCS ATG TGC AGY ACC AGT AA-3' R: 5'-CCG CRA TAT CRT TGG TGG TG-3'	543 bp	Heffernan et al.[19] ,Saladin et al.[22]
CTX-M group 1	F: 5'-CCC ATG GTT AAA AAA TCA CTG-3' R: 5'-CCG TTT CCG CTA TTA CAA AC-3'	891 bp	Heffernan et al.[19], Jeong et al.[23]
SHV	F: 5'-GCC GGG TTA TTC TTA TTT GTC GC-3' R: 5'-TCT TTC CGA TGC CGC CGC CAG TCA-3'	1007 bp	Heffernan et al.[19], Tenover and Rasheed[20]
TEM	F: 5'-GTA TCC GTC CAT GAG ACA ATA-3' R: 5'-TCC AAA GTA TAT ATG AGT AAA C-3'	966 bp	Heffernan et al.[19], Arpin et al.[21]

F: Forward primer; R: Reverse primer; S:G/C; Y:C/T; R:A/G; bp: Base pair

In PCR test to determine the CTX-M gene performed with CTX-M universal primers (Table 1); PCR mix consisted of 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2.5 µl dNTP mix (2 mM each), 1.5 Units (0.3 µl) Taq DNA polymerase, 13.5 µl ddH_2O and 3 µl template DNA. The thermocycler program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer binding at 54°C for 30 sec, and extension at 72°C for 1 min, and lastly a final extension step of 7 min was performed at 72°C.[8]

In PCR test for CTX-M group 1; the PCR reaction was performed with 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl forward primer (10 µM) and 0.5 µl reverse primer (10 µM)(*Table 1*), 2.5 µl dNTP mixture (2 mM each), 1.5 Units (0.3 µl) Taq DNA polymerase, 15 µl ddH_2O and 2.5 µl template DNA. The thermocycler program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and finally final extension at 72°C for 7 min[8].

In PCR test for the SHV gene; the PCR reaction was performed with 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM)(Table 1), 2.5 µl dNTP mixture (2 mM each), 1.5 U (0.3 µl) Taq DNA polymerase, 13.5 µl ddH_2O and 3 µl template DNA. The thermocycler program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of

denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 1 min, and finally a final elongation at 72°C for 7 min.[8]

In TEM gene specific PCR test; PCR mix contained 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM)(Table 1), 2.5 µl dNTP mixture (2 mM each), 1.5 U (0.3 µl) Taq DNA polymerase, 14 µl ddH_2O and 2.5 µl template DNA. The thermocycler program was as follow: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer binding at 48°C for 1 min, and extension at 72°C for 1 min, and finally a final elongation at 72°C for 10 min.[8]

PCR products were run in a prepared 1% agarose gel and a constant voltage of 85 V for 45 minutes. DNA bands were visualized and photographed by a gel imaging system (EDAS 290-Kodak) under ultraviolet light.

Susceptibility to Other Classes of Antibiotics

The susceptibility of ESBL-producing *E. coli* in our study to beta-lactam antibiotics and antibiotics belonging to other classes was investigated by agar disk diffusion test (ADDT) on MHA according to CLSI protocols.[18] The antibiotic disks, containing beta lactams, used in the test were: ampicillin (AMP, 10 µg), aztreonam (ATM, 30 µg), cefepime (FEP, 30 µg), cefpodoxime (CPD, 10 µg), ceftriaxone (CRO, 30 µg), cefuroxime (CXM, 30 µg), cephalothin (CEF, 30 µg), ceftiofur (FUR, 30 µg) and imipenem (IPM, 10 µg). Antibiotics from other classes tested were; chloramphenicol (CHL, 30), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), nalidixic acid (NAL, 30 µg), streptomycin (STR, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg) and tetracycline (TET, 30 µg)(Bioanalyse, Türkiye). An isolate resistant to at least one agent in three or more antibiotic classes was accepted as multidrug resistant isolate.

The evaluation of the infection zone diameters was performed according to the following criteria: Growth inhibition zone of AMP, CAZ, CHL, IPM, STR, GEN, SXT, KAN, FFC, TET, CPD, ENR and FUR discs were evaluated according to CLSI document VET01S-ED7[24]; zone of ATM, CIP, CRO, CTX and CXM and NAL discs were evaluated according to CLSI document M 100 ED34.[25]

RESULTS

As a result of the simultaneous culture of 32 farm milk samples in the study on MCA medium supplemented with 2 µg/ml cefotaxime or 2 µg/ml ceftazidime, *E. coli* suspicious colonies in red colour (lactose positive) were seen in both selective cultures of only one sample. After obtaining the pure culture of the isolate and performing identification tests, it was determined that the isolate was *E. coli*.

After the combined disk method, the *E. coli* isolate was confirmed to be ESBL producer. In the PCR performed for CTX-M, SHV and TEM, it was found that the isolate carried the CTX-M gene but did not posses the TEM and SHV genes. Then, in the PCR specific for CTX-M group 1, it was determined that the isolate carried the group 1 CTX-M gene.

In the ADDT performed to determine the susceptibility of the isolate to various antibiotics belonging to the beta lactam group and other classes of antibiotics, the isolate was found resistant to tested beta lactams (AMP, ATM, CAZ, CEF, CPD, CRO, CTX, FEP and FUR) except IPM, aminoglycosides (GEN, KAN and STR), phenicols (CHL and FFC), tetracyclines (TET) and folate pathway inhibitors (SXT) but sensitive to fluoroquinolones (CIP, ENR and NAL). The isolate possesed a multiple antibiotic resistance phenotype, due to its resistance to more than two classes of antibiotics.

DISCUSSION

In studies conducted in different parts of Türkiye, the presence of ESBL-producing *E. coli* in cattle milk tank samples was investigated. Kurekci et al.[13] identified ESBL-producing *E. coli* in 14 (22.6%) of 62 milk tank milk samples in Hatay province, and the highest proportion of isolates (12 out of 14 ESBL-producing *E. coli*) contained CTX-M group 1 genes (CTX-M-15 and CTX- M-1). Özpinar et al.[11] isolated a total of 23 (17.04%) ESBL-producing *E. coli* in 135 milk tank milk samples collected from farms in Istanbul, Kocaeli and Sakarya cities. Tepeli et al.[12] isolated ESBL-producing *E. coli* in 5 (29.41%) of 17 milk tank milk samples collected from Çanakkale city. In our study, only 1 (3.12%, 1/32) ESBL-producing *E. coli* was isolated from 32 farm milk mixture samples, which seems considerably low compared to the research results in the mentioned cities in Türkiye.[11-13] However, it is not entirely possible to compare our results with the results of the mentioned studies since in our study, a milk sample is a mixture of milks from cows in one farm, while in others it is most likely a mixture of milk from many farms.[11-13] Therefore, we can think that the reason for the low prevalence in our study is that the milk samples in the study were collected by different approach. In the studies performed with similar sampling method for milk to our study, ESBL-producing *E. coli* was not detected in 100 milk tank samples from 100 farms in Switzerland[26] and in 85 farm milk tanks in Çanakkale city in Türkiye.[14] Similarly, in a study in Colombia, ESBL-producing *E. coli* was detected in 3.3% of 120 milk samples taken from 120 farm milk tanks[27], similar to low level like our study. As a result, it will be possible to state that the probability of isolating ESBL-producing *E. coli*

in the tank milk sample may vary depending on how many farm milks that a tank milk contains.

In the present study, ESBL-producing *E. coli* isolated from milk was CTX-M type ESBL producer, not TEM or SHV type ESBL producer. Similarly, Kurekci et al.[13] identified 14 ESBL-producing *E. coli* in tank milk samples in Hatay province in Türkiye, and 12 out of them contained CTX-M group 1 genes (CTX-M-15 and CTX- M-1) and it was highest proportion among the ESBL genes they detected. Vazquez et al.[27] found that all four *E. coli* isolates from farm milk tanks in their study in Colombia carried the CTX-M group 1 gene. Moreover, studies worldwide show also that CTX-M genes have been detected more frequently in normal flora and pathogenic *E. coli* isolates in both humans and animals and *E. coli* isolates causing foodborne infections in human than TEM and SHV type ESBL genes.[28-31] In a study covering 3 years (July 2015 and June 2018) in India, Devi et al.[28] showed that the proportions of *E. coli* isolated from community-acquired infections in humans carrying only CTX-M gene were 78.9% in Year 1, 80.8% in Year 2 and 91.1% in Year 3. Heldall et al.[29] compared *E. coli* strains isolated from urinary tract infections in elderly humans in Sweden between 2003-2005 and 2008-2009 time periods and showed that the proportion of CTX-M beta-lactamase gene detection increased significantly. Park et al.[30] reported that 80 ESBL-producing *E. coli* strains isolated from humans with foodborne diarrhea carried CTX-M gene the most (79/80) among ESBL genes. Mandujano-Hernández et al.[31] reported in their review article that the CTX-M beta-lactamase gene is found to be more prevalent in domestic and farm animals compared to other ESBL genes.

In the present study, ESBL producing *E. coli* was found resistant to aminoglycosides (GEN, KAN and STR), phenicols (CHL and FFC), tetracyclines (TET) and folate pathway inhibitors (SXT) in addition to beta-lactams, therefore it was a multidrug resistant ESBL-producing *E. coli* isolate. Similarly, multidrug characteristics of ESBL producing *E. coli* isolates have been shown in many studies.[32-34] The tendency of ESBL producing *E. coli* to show multiple antibiotic resistance is attributed to the fact that they are encoded on common plasmids with resistance genes that confer resistance to other antibiotics. Therefore, widespread use of antibiotics increases the selection of these plasmids and causes their spread in Gram-negative bacteria, including *E. coli*.[32-34]

The increase in ESBL-producing bacteria in farm animals has been found to be associated with the increase in ESBL-producing bacteria in humans. The role of consumption of animal foods (milk, meat and eggs) in this relationship is also obvious.[35] Transmission of ESBL-producing *E. coli* into cow milk may be caused by mastitis or through environmental contamination during milking/transportation or human originated contamination during milking/processing. In this study, the milk samples were taken from farm milk containers at the milk collection center, so ESBL-producing *E. coli* we detected may have been caused by one of the mastitis, environmental or human originated contamination sources. Thus, determining the source of ESBL-producing *E. coli* detected in farm milk through more detailed studies will pave the way for the necessary measures to be taken to obtain better quality cow milk for human consumption.

In conclusion, the detection of low-level ESBL-producing *E. coli* in farm milk in Burdur province is a positive finding. However, continuous monitoring of ESBL-producing *E. coli* levels in farm milk in Burdur province is important for both animal and public health.

Ethics Committee Approval: This study was approved by the Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Date: 02.10.2024; Decision Number:1373).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – F.P.; Design – F.P.; Supervision – F.P.; Resources – F.P.; Data Collection and/or Processing – F P. and S.K.G; Analysis and/or Interpretation- F.P. and S.K.G.; Literature Search – F.P. ; Writing Manuscript – F.P. and S.K.G.; Critical Review – F.P. and S.K.G.

Declaration of Interests: The authors declare that there are no conflict of interest.

Funding: This research has not benefited from any financial resources. All experiments in the research were carried out in the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University.

Etik Komite Onayı: Bu çalışma Mehmet Akif Ersoy Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylanmıştır (Tarih: 02.10.2014, Karar No: 1373).

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Konsept – F.P.; Tasarım – F.P.; Denetim – F.P.; Kaynaklar – F.P.; Veri Toplama ve/veya İşleme – F.P. ve S.K.G; Analiz ve/veya Yorum-F.P. ve S.K.G.; Literatür Taraması – F.P.; Yazma – F.P. ve S.K.G.; Eleştirel İnceleme –

F.P. ve S.K.G.

Çıkar Çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

Finansal Destek: Bu araştırmada herhangi bir mali kaynaktan faydalanılmamıştır. Araştırmadaki tüm deneyler Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı laboratuvarında gerçekleştirildi.

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ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1676194

Insights into Lameness in Dairy Cattle: The Role of Body Condition Score and Lactation Dynamics

Süt Sığırlarında Topallık Üzerine İçgörüler: Vücut Kondisyon Skoru ve Laktasyon Dinamiklerinin Rolü

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ABSTRACT

Lameness is a significant welfare and economic issue in dairy farms, resulting in reduced milk yield, increased treatment costs, and higher culling rates. The present study aimed to investigate the relationships between lameness and animal-level factors including body condition score (BCS), lactation period (LP), and lactation number (LN) in 370 lactating Holstein cows. Lameness and BCS were assessed by observation, while LP and LN were obtained from farm records. Data were analyzed using chi-square and Fisher's-Freeman-Halton exact test to examine relationships between lameness scores and animal-level factors, and logistic regression was performed to reveal risk factors. Chi-square analysis revealed significant associations between lameness scores and both BCS (P = .010) and LN (P = .039), but not with LP (P > 0.05). Consistent with these results, logistic regression identified BCS as a significant predictor (P =.012); cows with a BCS of 4 had 3.72 times higher odds of lameness compared to those with a BCS of 2 (P = .037). Although LN was not significant overall (P = .195), cows in their second lactation showed significantly lower odds of lameness compared to first-lactation cows (P = .045). LP was not a significant predictor in the multivariate model (P = .746). Present findings emphasize the importance of monitoring BCS and LN to reduce lameness risks and improve herd health. Further studies are required to clarify the role of animal level factors in lameness.

Keywords: Body condition score, dairy cows, days in milk, lactation number, lameness

ÖZ

Topallık süt çiftliklerinde süt veriminde azalma, tedavi maliyetlerinde artış ve kesim oranlarında yükselmeye neden olan önemli bir refah ve ekonomik sorundur. Sunulan çalışmada 370 sağmal Holstein inekte topallık ile vücut kondisyon skoru (VKS), laktasyon periyodu (LP) ve laktasyon sayısı (LS) gibi hayvan düzeyindeki faktörler arasındaki ilişkilerin değerlendirilmesi amaçlandı. Topallık ve vücut kondisyon skorları gözlem yoluyla değerlendirilirken, laktasyon sayısı ve periyodu çiftlik kayıtlarından elde edildi. Topallık skorları ile hayvan düzeyindeki faktörler arasındaki ilişkileri incelemek için veriler ki-kare ve Fisher's-Freeman-Halton kesin testi kullanılarak analiz edildi ve risk faktörlerini belirlemek için ise lojistik regresyon uygulandı. Ki-kare analizinde, topallık skorları ile hem VKS (P = 0,010) hem de LS (P = 0,039) arasında anlamlı ilişkiler bulundu; LP ile ise anlamlı bir ilişki saptanmadı (P > 0,05). Bu sonuçlarla tutarlı olarak, lojistik regresyon analizinde VKS'nin anlamlı bir belirleyici olduğu saptandı (P = 0,012); VKS'si 4 olan ineklerin, VKS'si 2 olanlara kıyasla topallık görülme olasılığının 3,72 kat daha yüksek olduğu belirlendi (P = 0,037). LS genel olarak anlamlı bulunmamakla birlikte (P = 0,195), ikinci laktasyondaki ineklerde topallık görülme olasılığı, birinci laktasyondakilere göre anlamlı düzeyde daha düşük bulundu (P = 0,045). LP ise çok değişkenli modelde anlamlı bir risk faktörü olarak bulunmadı (P = 0,746). Mevcut bulgular, topallık risklerini azaltmak ve sürü sağlığını iyileştirmek için BCS ve LN'nin izlenmesinin önemini vurgulamaktadır. Hayvan düzeyindeki faktörlerin topallıktaki rolünü açıklığa kavuşturmak için daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Laktasyon sayısı, sığır, süt verim gün sayısı, topallık, vücut kondisyon skoru

Received/Geliş Tarihi: 14.04.2025
Accepted/Kabul Tarihi: 22.08.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Özturan YA, Akın İ. Insights into lameness in dairy cattle: the role of body condition score and lactation dynamics. *Vet Sci Pract*. 2025;20(2):64-70.

Atıf: Özturan YA, Akın İ. Süt sığırlarında topallık üzerine içgörüler: vücut kondisyon skoru ve laktasyon dinamiklerinin rolü. *Vet Sci Pract*. 2025;20(2):64-70.

INTRODUCTION

Lameness is widely recognized as one of the three most costly diseases affecting dairy herds, alongside mastitis and infertility.[1-4] The economic losses associated with lameness arise from decreased milk yield, reduced fertility, treatment costs, premature culling, and compromised cow welfare.[5-7] It is also associated with high treatment expenses and prolonged recovery periods.[7,8] Numerous studies have investigated methods to understand and mitigate the adverse effects of lameness in dairy cattle.[9-13]

Identifying the factors contributing to the high incidence of lameness, as well as the associated risk factors, is crucial for developing effective intervention strategies on dairy farms.[14] Previous studies have linked both animal- and farm-level risk factors to an increased incidence of lameness, with animal-level risks including parity, BCS, hock injuries, and milk yield, and farm-level risks encompassing management practices such as trimming intervals, footbath usage, herd size, flooring, and lying surface.[15-17]

In previous studies BCS is closely related to lameness incidence in dairy farms.[18,19] It has been hypothesized that low BCS contributes to claw horn lesions, potentially due to reduced digital cushion thickness, which is associated with poor body condition.[18] A BCS of ≤ 2 on a 0-to-5 scale is considered a significant risk factor, markedly increasing the likelihood of lameness.[19] Similarly, high-yielding cows are claimed to be more prone to lameness, which can lead to significant milk yield losses, estimated at approximately 350 kg over a 305-day LP.[20,21] While most research emphasizes the role of parity[22,23] and BCS[24,25] in lameness, there is a lack of detailed information on whether LP is an independent risk factor for lameness after adjusting for other variables such as BCS and LN. This issue is addressed in the present study.

This study aimed to investigate the relationships between lameness and BCS, LN, and LP in dairy cows, while also utilizing logistic regression analysis to identify significant risk factors and their contribution to lameness.

MATERIALS AND METHODS

Animals and Farm

The study included 370 lactating cows (n = 370) from a herd of 500 Holstein cattle. The cows were milked twice daily in a double herringbone milking parlor. They were housed in free-stall barns equipped with concrete stalls covered with mattresses in the lying areas. The walking alleys within the pens had grooved concrete floors and were cleaned by automatic scrapers. All walkways leading to and from the milking barn and holding pen, spanning approximately 75-85 meters, were covered with rubber mats. The cows were fed a total mixed ration comprising 45% concentrate (including soybean meal, cornmeal, cottonseed, and canola) and 55% forage (wheat straw, alfalfa hay, haylage, and corn silage). Routine claw trimming interval was once in 7 months according to the farm protocol by a claw trimmer, and all cows underwent claw trimming during their dry-off period. Although the routine claw trimming interval was 7 months—longer than the commonly recommended 3-6 months—this may have influenced the lameness prevalence observed in this study. The study was approved by the Adnan Menderes University Local Ethics Committee (Date: February 6, 2025, Approval number: 64583101/2025/034).

Body Condition Score Assessment and Lactation Related Data Collection

Body condition scoring was performed from the rear view of the cows while they were feeding in their pens. The BCS was determined as outlined in a previous study, where a score of 1 indicated an undercondition cow, 3 ideal condition and a score of 5 indicated an overcondition.[26] Data on LN and days in milk (DIM) were obtained from the farm's records.

Lameness Assessment

Lameness assessments were conducted on all cows following the afternoon milking session as they exited the milking parlor through an alley, enabling individual observation. The lameness scoring system was adapted from the Agriculture and Horticulture Development Board (AHDB) guidelines, where a score of 0 indicated a cow with no signs of lameness.[27] Lameness scores (LS) from 1 to 3 represented increasing levels of lameness severity, categorized as follows: LS1 for mild lame cows, LS2 for moderate lame cows, and LS3 for severe lame cows.

Categorization of Lactation Period and Lactation Number

Cows' DIM were classified into three categories based on their lactation cycle: early lactation (0-120 DIM), mid-lactation (121-240 DIM), and late lactation (241-360 DIM), as described in a previous study.[28] Lactation numbers equal to or greater than 5 were categorized as one group, while cows with LN smaller than 5 were left uncategorized.

Statistical Analysis

Statistical analyses were performed using SPSS® 22 statistical software (IBM SPSS Corp., Armonk, NY, USA). The relationships between lameness scores and BCS, LP, and LN were analyzed using the chi-square test. When the assumptions of the chi-square test were not met, the Monte Carlo simulation method was employed to provide more accurate p-values with 95% confidence intervals (CI). Fisher's-Freeman-Halton exact test was applied when the expected count for any cell was less than 5. Binary logistic regression analysis was performed to evaluate the association between lameness status (lame vs. not lame) and potential predictor variables, including BCS, LN, and LP. Lameness scores were dichotomized, with cows classified as either not lame (score = 0; reference category) or lame

(scores = 1, 2, or 3). All predictor variables were treated as categorical and were dummy coded for inclusion in the model. The clinically relevant category was designated as the reference group: BCS = 2, LN = 1, and LN = Early lactation. Odds ratios (Exp(β)) were used to interpret the strength and direction of associations. The overall significance of each factor was assessed using the likelihood ratio test, and a *P*-value < .05 was considered statistically significant. Prior to analysis, all predictor variables were assessed for multicollinearity using the variance inflation factor (VIF), with values < 2.0 considered acceptable. The model's fit was evaluated using the Hosmer-Lemeshow goodness-of-fit statistic, with a *P*-value of > 0.05 considered statistically significant.

RESULTS

The incidence of lameness in lactating cows in our study was 31.08% (115 out of 370 cows), which is higher than the prevalence reported in comparable freestall-housed Holstein herds, where previous studies have documented rates ranging from 21% to 25%.[22,23] The relationships between lameness and animal-level factors, including BCS, LN, and LP, are detailed in Tables 1, 2, and 3, respectively. The results of the logistic regression analysis for these animal-level predictors are provided in Table 4.

Table 1. Relationship Between Body Condition Scores and Lameness Scores.

		LS (%)				Total (%)
		0	1	2	3	
BCS	2	40 (15.7)[a,b]	4 (5)[a]	8 (29.6)[b]	2 (25)[a,b]	54 (14.6)
	3	199 (78.1)[a]	74 (92.5)[b]	17 (63)[a]	6 (75)[a,b]	296 (80)
	4	16 (6.3)	2 (2.5)	2 (7.4)	0	20 (5.4)
Total		255 (68.9)	80 (21.6)	27 (7.3)	8 (2.2)	370 (100)

BCS: Body condition score; LS: Lameness score. Different lowercase letters (a, b) within the same row indicate statistically significant differences between groups (*P* = .010; .008-.013 95% Confidence Interval).

Table 2. Relationship Between Lactation Number and Lameness Score of Cows.

		LS (%)				Total (%)
		0	1	2	3	
LN	1	102 (40)	26 (32.5)	4 (14.8)	2 (25)	134 (36.2)
	2	70 (27.4)	22 (27.5)	8 (29.6)	4 (50)	104 (28.1)
	3	40 (15.7)	16 (20)	4 (14.8)	0	60 (16.2)
	4	29 (11.4)[a]	10 (12.5)[a,b]	9 (33.4)[b]	0[a,b]	48 (13)
	≥ 5	14 (5.5)	6 (7.5)	2 (7.4)	2 (25)	24 (6.5)
Total		255 (68.9)	80 (21.6)	27 (7.4)	8 (2.1)	370 (100)

LN: Lactation number; LS: Lameness score. Different lowercase letters (a, b) within the same row indicate statistically significant differences between groups (*P* = .039; .034-.044 95% Confidence Interval).

Table 3. Relationship Between Lactation Period and Lameness Score of Cows.

		LS (%)				Total (%)
		0	1	2	3	
LP	Early	55 (21.6)	14 (17.5)	11 (40.7)	2 (25)	82 (22.2)
	Mid	54 (21.2)	16 (20)	4 (14.8)	2 (25)	76 (20.5)
	Late	146 (57.2)	50 (62.5)	12 (44.5)	4 (50)	212 (57.3)
Total		255 (68.9)	80 (21.6)	27 (7.3)	8 (2.2)	370 (100)

LP: Lactation period; LS: Lameness score (*P* = .354; .342-.366 95% Confidence Interval).

Table 4. Logistic Regression Analysis of Lameness Scores Based on Body Condition Score, Lactation Number, and Lactation Period.

Factors	no.	*P*	β	SE	Exp (β)
BCS:		.012			
2	290		0.000	0.000	1.000
3	60	.492	0.409	0.596	1.505
4	20	.037	1.314	0.630	3.723
LN:		.195			
1	134		0.000	0.000	1.000
2	104	.045	-.862	0.467	0.422
3	60	.156	-.689	0.486	0.502
4	48	.479	-.356	0.503	0.701
5	24	.677	-.216	0.518	0.806
LP:		.746			
Early	82		0.000	0.000	1.000
Mid	76	.570	0.168	0.296	1.183
Late	212	.752	-0.097	0.306	0.908

BCS: Body condition score; LN: Lactation number; LP: Lactation period; β: Estimated coefficient; SE: Standard error; Exp (β): Odds ratio.

Relationship Between Body Condition Score and Lameness Score

In all BCS groups, cows with lameness score of 0 were the most prevalent (n: 40, 199, and 16 for BCS 2, 3, and 4, respectively). Among lameness score groups, cows with BCS 3 were predominant (LS0: 199, LS1: 74, LS2: 17, LS3: 6). A statistically significant difference was identified between mild lame (LS1) and moderate lame (LS2) cows within the BCS 2 group. Similarly, within the BCS 3 group, a significant difference was observed between mild lame (LS1) and moderate lame (LS2) cows (*P* = .010; .008-.013 95%CI; Table 1).

Relationship Between Lactation Number and Lameness Score

Cows classified as not lame (LS0, 255 cows) comprised the largest group across all lactation numbers (Table 2). Specifically, cows in their first lactation (LN1) were the most represented in both the not lame (LS0, 102 cows) and mild lame (LS1, 26 cows) groups. Among lame cows (LS2), the highest proportions were observed in cows in their fourth (LN4, 9 cows) lactations. For severe lame cows (LS3), cows in their second lactation (LN2, 4 cows) had the highest proportion (Table 2).

In cows in their fourth lactation, a significant difference was observed between non-lame (LS0, 29 cows) and moderately lame cows (LS2, 9 cows) (*P* = .039; .034-.044 95%CI; Table 2).

Relationship Between Lactation Period and Lameness Score

In all lactation periods (early, mid, and late), cows with lameness score of 0 had the highest proportions (n: 55, 54, and 146, respectively). Late-lactation cows had the highest proportion across all lameness scores (LS0: 146, LS1: 50, LS2: 12, LS3: 8 cows). No statistically significant differences were observed between lameness scores and lactation periods (*P* > .05; Table 3).

Logistic Regression Analysis of Animal Level Risk Factors for Lameness

Logistic regression analysis was conducted to determine the association between lameness and animal-level factors including BCS, LN, and LP. Among these, BCS emerged as a significant predictor of lameness (*P* = .012). Cows with a BCS of 4 had 3.72 times greater odds of being lame compared to those with a BCS of 2 (*P* = .037), while the difference between BCS 3 and BCS 2 was not statistically significant (*P* = .492). Although LN was not statistically significant overall (*P* = .195), cows in their second lactation demonstrated a significantly reduced likelihood of lameness compared to first-lactation cows (OR = 0.422; *P* = .045). No significant associations were found for higher lactation numbers. Similarly, LP was not significantly associated with lameness (*P* = .746), and comparisons between mid or late lactation and early lactation did not yield significant differences (*P* > .05; Table 4).

DISCUSSION

The findings of this study provide valuable insights into the relationships between lameness and animal-level factors, including BCS, LN, and LP in dairy cows. These results highlight key predictors of lameness, offering valuable guidance for targeted management strategies to mitigate its occurrence and associated economic losses.

Previous studies have demonstrated that maintaining a BCS above 2.5 out of 5 significantly reduces the risk of claw horn lesions requiring treatment.[19] An 8-year longitudinal study similarly reported a higher likelihood of lameness in cattle with a BCS below 2.[29] Both studies suggested a positive association between BCS and digital cushion thickness, with higher BCS linked to thicker digital cushions that help dissipate concussive forces during claw strikes, thereby reducing the risk of claw horn lesions.[18] In our study, significant differences were observed between mildly and moderately lame cows with a BCS of 2 (Table 1), supporting the notion that underconditioned cows are more predisposed to lameness. Consistent with this, earlier research also reported a higher prevalence of claw diseases in cows with a BCS of 3-4, whereas non-infectious claw disorders were less common in cows with a BCS below 3.[30] Importantly, logistic regression analysis revealed that BCS was a significant predictor of lameness. Cows with a BCS of 4 had significantly greater odds of being lame compared to

those with a BCS of 2, indicating that overconditioning may also elevate lameness risk (Table 4). This association may be influenced by farm-specific management or environmental conditions that impact hoof health in overconditioned animals. These findings emphasize the importance of maintaining cows within an optimal BCS range, as both underconditioning and overconditioning appear detrimental to locomotor health. Further research should aim to clarify the pathophysiological mechanisms linking high BCS to lameness, particularly in relation to biomechanical load distribution, altered locomotion, and subclinical claw pathology. A limitation of this study is the lack of specific records on the causes of lameness or the association between claw diseases and BCS. Future research should aim to investigate this relationship to provide a more comprehensive understanding of the interplay between BCS, claw diseases, and lameness.

Lactation number has been previously associated with lameness, with several studies reporting increased lameness risk in cows with higher parity, particularly during the fourth lactation.[17,31,32] In our study, descriptive comparisons revealed differences in lameness prevalence across lactation numbers (Table 2); however, logistic regression analysis did not identify LN as a statistically significant predictor overall (Table 4). Notably, cows in their second lactation had significantly lower odds of lameness compared to first-lactation cows, while no significant associations were observed for higher lactation numbers (Table 4). This finding may suggest that younger, primiparous cows face unique physiological or management-related stressors that predispose them to lameness, whereas more mature cows in their second lactation may benefit from improved metabolic stability or hoof resilience. Future studies with larger sample sizes and a more balanced distribution of cows across lactation numbers are warranted to clarify the relationship between parity and lameness risk.

Lactation period is closely intertwined with BCS dynamics. BCS fluctuates during lactation, with Holsteins in early lactation averaging a BCS of 3, followed by an increase during mid-lactation, and stabilizing at approximately 3.5 during late lactation.[33] Most prior studies investigating the relationship between lameness and LP have focused on the dry period.[25,34] However, our study examined early, mid, and late lactation periods to assess their association with lameness cases. No statistically significant differences were found between LP and lameness, either through chi-square or logistic regression analyses (Tables 3 and 4). While LP can influence physical stress and metabolic demands, its effect on lameness may be less direct compared to other factors such as farm management protocols.[35] A notable limitation of this study is that the results are derived from a single dairy farm. Expanding the sample size and incorporating multi-farm data could yield more robust conclusions.

To further clarify whether BCS, LN, and LP are associated with differences in lameness prevalence, we examined the percentage of lame cows within each category. Chi-square analysis revealed significant differences in lameness distribution across BCS categories. Specifically, the prevalence of lameness was higher in cows with BCS 3 and 4 compared to those with BCS 2. For LN, a significant difference was observed between not lame and moderately lame cows in the fourth lactation group, suggesting parity may influence lameness occurrence. In contrast, the distribution of lameness was not significantly different across lactation period groups. These results demonstrate that both BCS and LN are associated with variation in lameness prevalence and highlight the importance of monitoring these animal-level factors for early detection and prevention.

In this study, we employed both chi-square tests and logistic regression to investigate the relationships between lameness and animal-level factors such as BCS, LN, and LP. Chi-square tests were used to identify simple associations and detect statistically significant differences between categorical variables, such as lameness scores and BCS and LN categories, providing an overview of potential relationships. To address these limitations, in this study, logistic regression analysis was utilized to evaluate the significant predictors of lameness while adjusting for other factors in the model. The complementary use of these statistical methods ensures a robust analysis and a more comprehensive understanding of the factors influencing lameness in dairy cows. A limitation of this study is the relatively small number of animals in certain categories, particularly cows with a BCS of 4 and those in the ≥5 LN group. While statistically significant findings were observed in these subgroups, the limited sample sizes may reduce the generalizability and robustness of these associations. Combining categories was avoided to preserve the biological relevance of each classification. Future studies with larger and more balanced sample distributions are warranted to confirm and expand upon these findings. One potential factor influencing the overall lameness prevalence observed in this study may be the relatively long claw trimming interval of 7 months. Routine trimming at shorter intervals (3–6 months) is typically recommended to prevent claw overgrowth and reduce lameness prevalence.[36] As all cows were managed under the same schedule, this limitation applies uniformly to the study population but should be considered in interpreting the present study findings.

As a result, this study highlights the complex interplay between lameness and key animal-level factors such as BCS, LN, and LP in dairy cows. While significant associations were observed between BCS and lameness in certain categories, logistic regression confirmed BCS as a significant predictor only for cows with a BCS of 4. In contrast, higher lactation numbers were significantly associated with increased lameness severity, emphasizing the need for targeted management strategies for older and multiparous cows. Although no significant relationship was found between LP and lameness, future studies involving larger sample sizes and multi-farm settings are warranted to explore this association further. These findings underscore the importance of individualized and stage-specific interventions in managing lameness, particularly for cows with specific BCS and in late lactation. Incorporating preventive measures and refining farm management practices may mitigate the impact of lameness, improve animal welfare, and enhance the economic sustainability of dairy farms. Additionally, longitudinal follow-up and multi-farm or regional studies are recommended to improve the generalizability of the findings.

Ethics Committee Approval: The study was approved by the Animal Experiments Local Ethics Committee of Aydin Adnan Menderes University (Date: February 6, 2025, Approval number: 64583101/2025/034).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Y.A.O.; Design – Y.A.O.; Supervision – I.A.; Resources – Y.A.O., I.A.; Data Collection and/or Processing – Y.A.O.; Analysis and/or Interpretation: Y.A.O., I.A.; Literature Search – Y.A.O.; Writing Manuscript – Y.A.O.; Critical Review – Y.A.O., I.A.

Declaration of Interests: The authors declare no conflict of interest.

Funding: The authors declared they have no financial support for this study.

Etik Komite Onayı: Sunulan çalışma Aydın Adnan Menderes Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylanmıştır (Tarih: 6 Şubat 2025, Onay No: 64583101/2025/034).

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Kavramsallaştırma – Y.A.O.; Tasarım – Y.A.O.; Denetim – I.A.; Kaynaklar – Y.A.O., I.A.; Veri Toplama ve/veya İşleme – Y.A.O.; Analiz ve/veya Yorumlama – Y.A.O., I.A.; Literatür Taraması – Y.A.O.; Makale Yazımı – Y.A.O.; Eleştirel İnceleme – Y.A.O., I.A.

Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemektedir.

Finansal Destek: Yazarlar bu çalışma için finansal destek almadıklarını beyan etmişlerdir.

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ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1675127

Investigation of the Effects of Chrysin Against Azithromycin-Induced Heart Damage in Rats

Sıçanlarda Azitromisin ile Oluşturulan Kalp Hasarına Karşı Krisin'in Etkilerinin Araştırılması

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ABSTRACT

Azithromycin (AZM) is macrolide antibiotic used to treat infections of the upper and lower respiratory tract. In addition to its therapeutic effects, it has adverse effects such as cardiac and oxidative damage. Chrysin (CHR), which is found in propolis and various plants, is a natural flavonoid known for its antioxidant properties. In this study, we investigated the protective effect of CHR against cardiac damage caused by AZM, a broad-spectrum antibiotic. For this purpose, twenty-eight female rats were divided into four groups: Control, CHR, AZM, AZM+CHR. AZM (200 mg/kg) and CHR (50 mg/kg) were administered orally once daily for seven days. Cardiac markers and oxidative stress parameters were analyzed to determine heart tissue damage. Histopathological analyses were performed to detect tissue damage and structural changes. According to the data obtained from these analyses, AZM increased lactate dehydrogenase (LDH) and creatine kinase-myocardial band (CK-MB) activities and cardiac troponin-I (cTn-I) levels in the heart tissue. AZM toxication significantly increased malondialdehyde (MDA) levels while reducing the activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) activities and glutathione (GSH) levels. AZM+CHR treatment decreased cardiac tissue cardiac markers (LDH, CK-MB, and cTn-I). In addition, CHR treatment together with AZM decreased MDA levels and increased GSH levels and GPx, SOD, and CAT activities. When the findings were evaluated together, it was determined that AZM caused heart damage by increasing cardiac markers and oxidative stress, while CHR supplementation reduced the damage by bringing these parameters closer to normal.

Keywords: Azithromycin, chrysin, heart, oxidative stress, rat

ÖZ

Azitromisin (AZM), üst ve alt solunum yolu enfeksiyonlarının tedavisinde kullanılan bir makrolid antibiyotiktir. Terapötik etkilerinin yanında kardiyak ve oksidatif hasar gibi olumsuz etkilere sahiptir. Propoliste ve çeşitli bitkilerde bulunan Krisin (CHR), antioksidan özelliğiyle bilinen doğal bir flavonoiddir. Bu çalışmada, geniş spektrumlu bir antibiyotik olan AZM'nin neden olduğu kalp hasarına karşı CHR'nin koruyucu etkisi araştırıldı. Bu amaçla, yirmi sekiz dişi sıçan Kontrol, CHR, AZM, AZM+CHR olmak üzere dört gruba ayrıldı. AZM (200 mg/kg) ve CHR (50 mg/kg) yedi gün boyunca günde bir kez oral yoldan uygulandı. Kalp dokusunda hasarı belirlemek için kardiyak belirteçler ve oksidatif stres parametreleri analiz edildi. Doku hasarını ve yapısal değişiklikleri tespit etmek için histopatolojik analizler yapıldı. Bu analizler sonucunda elde edilen verilere göre AZM, kalp dokusunda laktat dehidrogenaz (LDH), kreatin kinaz-miyokardiyal bant (CK-MB) aktiviteleri ve kardiyak troponin-I (cTn-I) seviyesini artırdı. AZM toksikasyonu, süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx), katalaz (CAT) aktiviteleri ve glutatyon (GSH) seviyeleri gibi antioksidan enzimlerin aktivitelerini azaltırken, malondialdehit (MDA) seviyelerini önemli ölçüde artırmıştır. AZM+CHR tedavisinin kalp dokusu kardiyak belirteçlerinde (LDH, CK-MB, cTn-I) azalma gösterdiği tespit edildi. Ayrıca, CHR tedavisinin AZM ile birlikte uygulanması MDA düzeyini düşürmüş ve GSH düzeyini ve GPx, SOD ve CAT aktivitelerini artırmıştır. Elde edilen bulgular birlikte değerlendirildiğinde, AZM'nin kardiyak belirteçleri ve oksidatif stresi artırarak kalp hasarına neden olduğu, CHR destekleyici tedavisinin ise bu parametreleri normale yakınlaştırarak hasarı azalttığı tespit edildi.

Anahtar Kelimeler: Azitromisin, kalp, krisin, oksidatif stres, sıçan

Received/Geliş Tarihi: 13.04.2025
Accepted/Kabul Tarihi: 13.06.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Aygörmez S, Dalkılınç E, Akaras N, Maraşlı Ş. Investigation of the effects of chrysin against azithromycin-induced heart damage in rats. *Vet Sci Pract*. 2025;20(2):71-77.

Atıf: Aygörmez S, Dalkılınç E, Akaras N, Maraşlı Ş. Sıçanlarda azitromisin ile oluşturulan kalp hasarına karşı krisin'in etkilerinin araştırılması. *Vet Sci Pract*. 2025;20(2):71-77.

INTRODUCTION

Azithromycin (AZM) is a effectively used broad-spectrum antibiotic of the macrolide group.[1,2] It has been reported have broad antimicrobial activity against both aerobic and anaerobic bacteria.[3,4] The Food and Drug Administration (FDA) reported that it can be used in respiratory infections.[2] In 2012, the FDA warned that AZM carries fatal cardiovascular risks. In 2013, it stated that AZM could cause potentially irregular heart rhythms in electrical activity of the heart.[1,5] Wei et al.[6] They examined the effects of AZM and reported that it caused cardiovascular malformations as a result of their findings. Different studies have stated that AZM causes heart damage, but the reasons underlying this damage mechanism have not been fully elucidated.[1,7] In addition, it has been reported that the risk of cardiovascular death increases in patients treated with AZM.[2] AZM administration is believed to increase the production reactive oxygen species (ROS) in heart tissue, and increased ROS triggers oxidative stress and myocardial tissue apoptosis, which increases heart damage.[1,2,8] The increase in ROS production is balanced by glutathione (GSH).[9] Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) also contribute to the process by neutralizing ROS and strengthening the defense system. Insufficiency of the mentioned antioxidants or constantly triggered production of ROS leads to disruption of the antioxidant-oxidant balance.[3] It has been reported that plant-based flavonoid and phenolic compounds, which are frequently used in alternative medicine and included in treatment protocols, prevent the development of many diseases.[10]

Chrysin (CHR) is a natural flavonoid found in plants including propolis, honey, shell of some walnut species, passion flower, wild Himalayan pear and bitter melon, used for therapeutic purposes.[11,12] Due to the presence of hydroxyl groups in the seventh-fifth positions, it is effective in eliminating free radicals and preventing the formation of oxidative stress.[13-15] In different studies where CHR was used as a supportive treatment, its antioxidant, antiapoptotic and anti-inflammatory properties have been determined and it has been stated it is effective in reducing or preventing many tissue damage thanks to these properties.[16-18]

The aim of the study was to investigate the effects of CHR on cardiac damage caused by AZM, which is frequently used in treatment.

MATERIALS AND METHODS

Chemicals

AZM (Azitro Tablet 500 mg, Tekirdağ) and CHR (Sigma, Cas. No: 480-40-0, 97% purity) were obtained commercially. Other chemicals were of analytical grade and were supplied by Sigma.

Ethical Approval

Ethical approval was obtained from the Atatürk University Animal Experiments Local Ethics Committee (Date: 31.01.2025, Approval No: 2025/01/22).

Groups and Experimental Procedures

Twenty-eight female *Sprague-Dawley* rats (220-250 g) were obtained from Atatürk University Experimental Research and Application Center (ATADEM) (Erzurum/Türkiye). The rats were housed in cages at 24-25 °C with a 12-hours light-dark cycle. Groups (n = 7):

1) Control: Orally administered saline for seven days.
2) Chrysin (CHR): 50 mg/kg CHR administered orally for seven days.[16]
3) Azithromycin (AZM): 200 mg/kg AZM administered orally for seven days.[19]
4) Azithromycin+Chrysin (AZM+CHR): 50 mg/kg CHR administered half an hour after 200 mg/kg AZM for seven days.

After the treatments were completed (day 8), rats were decapitated under light sevoflurane (Sevorane®; Queenborough, UK) anesthesia and heart tissue was collected. A portion of the heart tissue was stored at −80 °C for biochemical examination, while the rest was stored in 10% formaldehyde solution for histological examination.

Analysis of Cardiac Markers

The lactate dehydrogenase (LDH) and creatine kinase-myocardial band (CK-MB) activities and cardiac troponin-I (cTn-I) level were determined by using rat ELISA kit of Sunred Biological Technology (Shanghai, China, LDH Cat. No: 201-11-0531, CK-MB Cat. No: 201-11-0312 and cTn-I Cat. No: 201-11-0640). Analysis was performed by ELISA Plate Reader (Bio-Tek, Winooski, VT) according to the instruction of manufacturer.

Oxidative Stress Analyses

Heart tissue was homogenized with 1.15% potassium chloride (KCl) for GSH and malondialdehyde (MDA) levels and CAT, glutathione peroxidase (GPx), SOD activities, the supernatant was obtained by centrifugation. The absorbance of the color formed by the thiobarbituric acid reaction at 532 nm was measured to determine the level of lipid peroxidation in heart tissue.[20] GSH levels and GPx, CAT, SOD activities and were analyzed to determine the antioxidant status. CAT activity was analyzed according to the method of Aebi[21], SOD activity by Sun et al.[22], GPx activity by Lawrence and Burk[23], and GSH level by Sedlak and

Lindsay.[24] Total protein content of heart tissue was determined according to the method of Lowry et al.[25]

Histopathological Analysis

Heart tissue from all groups were fixed in 10% neutral-buffered formalin for fixative purposes for 48 h. According to routine paraffin tissue processing procedures, the tissues were dehydrated in increasing alcohol levels and cleared in xylene. Then, 5 µm thick sections were obtained from paraffin-embedded tissues and stained with H&E for histopathological evaluation. Images from stained preparations were evaluated using a binocular Olympus Cx43 light microscope (Olympus Inc., Tokyo, Japan) and EP50 camera (Olympus Inc., Tokyo, Japan).

Statistical Analysis

Data obtained at the end of the study were statistically analyzed using SPSS 20.0 (IBM SPSS Corp., Armonk, NY, USA). Data was presented as the mean ± standard error (SEM). Tukey's post hoc tests and one-way analysis of variance (ANOVA) were used for multiple comparisons. Statistical significance was determined at $P < .05$.

RESULTS

Effect of AZM and CHR on LDH, cTn-I and CK-MB Parameters in Heart Tissue

The effects of AZM on rat heart tissue examined, and the findings are shown in Figure 1. LDH (Figure 1A), CK-MB (Figure 1B) activities and cTn-I (Figure 1C) levels evaluated to determine cardiac markers in the heart tissue. According to the obtained data, it determined that cTn-I levels and CK-MB, LDH activities in the AZM toxication increased compared to that in the control and CHR groups ($P < .05$) and that AZM+CHR treatment was effective in bringing these markers to levels close to that in the control group.

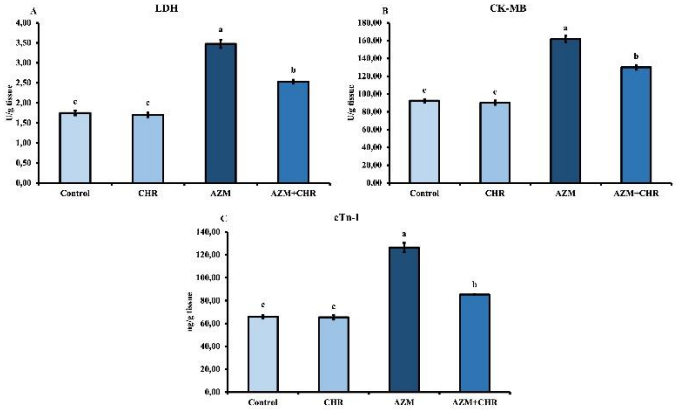

Figure 1: Effects of AZM and CHR applications on LDH (A) and CK-MB (B) activities and cTn-I (C) levels in rat heart tissue. Values for each group are presented as the mean ± SEM. Different letters in columns (a-b-c) indicate differences between groups ($P < .05$). (AZM: Azithromycin, CHR: Chrysin, LDH: Lactate dehydrogenase, CK-MB: Creatine kinase-myocardial band, cTn-I: Cardiac troponin-I).

Effect of AZM and CHR on Oxidative Stress Parameters in Heart Tissue

In the heart tissue, MDA (Figure 2A), GSH (Figure 2B) levels and GPx (Figure 2C), SOD (Figure 2D) and CAT (Figure 2E) activities evaluated. MDA levels were higher in the AZM toxication than in increased compared to the control and CHR groups ($P < .05$), whereas GPx, CAT, SOD activities and GSH levels decreased ($P < .05$). Supportive CHR treatment together with AZM decreased MDA and increased CAT, SOD, GPx activities, GSH levels, thus strengthening antioxidant defense system.

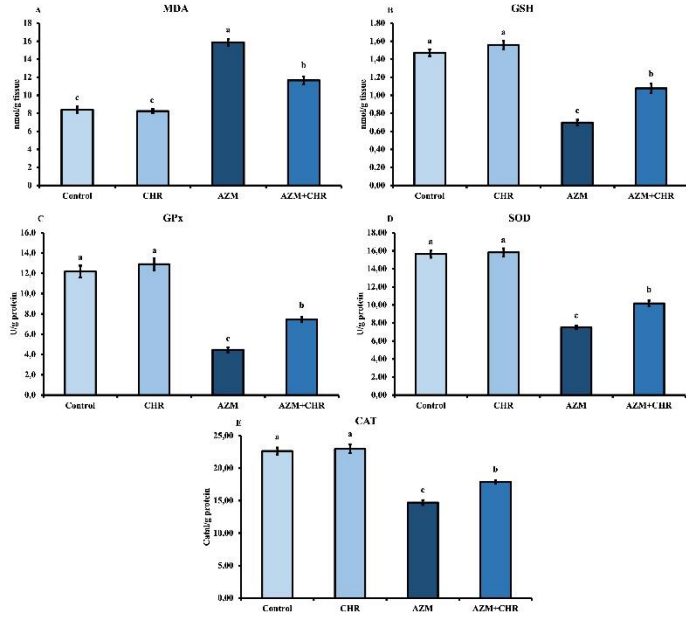

Figure 2: Effects of AZM and CHR applications on MDA (A) and GSH (B) levels and GPx (C), SOD (D) and CAT (E) activities in rat heart tissue. Values for each group are given as mean ± SEM. Different letters in the columns (a-b-c) indicate differences in groups ($P < .05$). (AZM: Azithromycin, CHR: Chrysin, MDA: Malondialdehyde, GSH: Glutathione, GPx: Glutathione peroxidase, SOD: Superoxide dismutase, CAT: Catalase).

Histopathological Results

The histopathological results in the heart tissue sections of the control, CHR, AZM groups are shown in Figure 3. In the control and CHR groups, myocardial muscle fiber branching and organization showed a normal histological alignment. The sarcolemma of cardiomyocytes in the control and CHR groups was regular, single-nucleated, and centrally located. The images of the AZM group showed distinct pathological changes. As a result of AZM application, scattered organization due to separation was observed in the muscle fibers. Eosinophilic changes and pyknotic nuclei were observed in the cytoplasm of cardiomyocytes in this group. Additionally increased vascular congestion and leukocyte infiltration were observed. In the group where CHR was applied together with AZM, cardiomyocytes showed a more

regular alignment and fewer gaps between muscle fibers, indicating that it was comparable to the control. In addition, the number of congested vessels and inflammatory cells was significantly reduced.

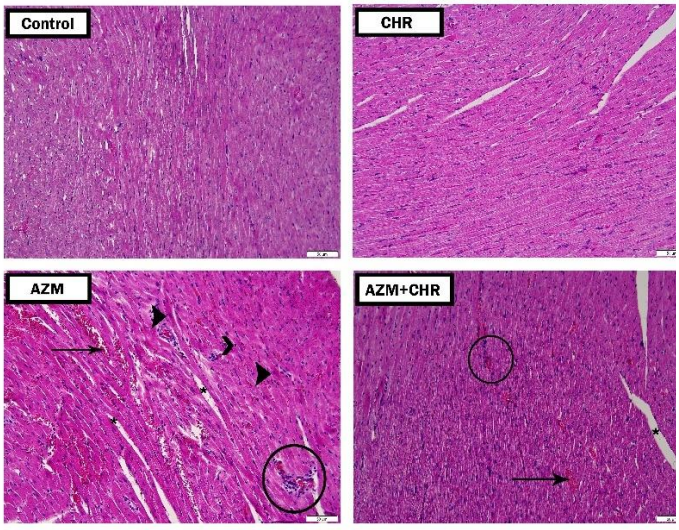

Figure 3. Photomicrographs of heart tissues stained with H&E from the control and experimental groups. The heart tissues from the control and CHR groups showed a normal structure. The heart from the AZM group shows clearly separated muscle fibers (star), inflammatory cells (circle), cardiomyocytes with eosinophilic cytoplasm (arrowhead), cardiomyocytes with pyknotic nuclei (arrowhead), and vascular congestion (arrow) in the myocardial layer. The AZM+CHR group showed recovery similar to that of control group, except for very slightly separated muscle fibers (star), decreased inflammatory cells (circle), and congestion (arrow). (H&E, x200). (AZM: Azithromycin, CHR: Chrysin).

DISCUSSION

AZM, which is used in the treatment of bacterial infections, causes tissue damage, especially in the heart tissue. This damage is associated with the activation of the damage pathways triggered by oxidative stress due to increased ROS production. Therefore, in this study, we investigated the effects of CHR, a flavonoid, on AZM-induced heart damage.

When there is a sudden decrease in coronary blood flow, myocardial damage occurs.[7] LDH, one of the cardiac markers, is released into the blood from the myocardium damaged by AZM by inducing ischemia.[2] It has been reported that the mitochondrial toxicity of AZM is associated with cardiac side effects.[26] CK-MB, LDH activities, cTn-I levels are important markers of cardiac damage.[27] In the current study, it was found AZM administration increased cTn-I levels and LDH, CK-MB activities in the heart tissue of rats, and as a result, caused damage to the heart tissue. This increase was supported by histopathological examination of the heart tissue. Studies have reported that AZM application causes damage by increasing LDH and CK-MB activities.[2,28] Different chemical agents increase cTn-I levels in heart tissue.[29] It was found that the application of CHR, a natural flavonoid, together with AZM reduces cTn-I levels with increasing LDH and CK-MB activities in heart tissue. Studies show that CHR has the ability to increase antioxidant activities against different toxic agents and reduce lipid peroxidation and prevent tissue damage.[12,16]

AZM, is a broad-spectrum antibiotic that, causes death in myocardial tissue via oxidative stress.[2] Oxidative stress occurs through the production of ROS such as superoxide anion radicals and peroxides.[30-32] ROS causes peroxidation of unsaturated fatty acids in cell membranes, damage to nucleic acids and denaturation of proteins. Thus, it leads to deterioration of cell structure and loss of tissue function.[33-37] Free radicals and an impaired antioxidant defense system cause redox imbalance.[38] Non-enzymatic (GSH) and enzymatic (GPx, SOD, CAT) antioxidants are important substances in defense against ROS.[39-41] GSH, a powerful antioxidant compound, helps maintain the redox state in cells.[12,39,42] SOD, GPx, CAT are antioxidant enzymes that provide antioxidant defense in the body.[14,43,44] SOD plays a role in the scavenging of superoxide radicals, CAT plays a role in the decomposition of hydrogen peroxide (H_2O_2) into molecular oxygen and water.[12,45] Another antioxidant defense system, GPx, has been reported play a role in the neutralization of cytotoxic lipid peroxides H_2O_2.[46] When ROS production and lipid peroxidation increase, mitochondrial activity is affected. It has been stated that ATP synthesis in the electron transport chain in mitochondria is negatively affected, causing damage to the cell.[47] MDA is a polyunsaturated fatty acid peroxidation product an indicator of oxidative stress.[27] Antioxidants reduce cellular damage caused by the interaction of protein, DNA, and lipid molecules with ROS.[48] In the present study, it was determined that in the damage induced by AZM in the heart tissue, MDA increased, GSH level, and SOD, GPx, CAT enzyme activities decreased which oxidative stress developed, development of oxidative stress caused tissue damage. El-Shitany and El-Desoky[1] examined heart damage in rats treated with AZM and reported that ROS production increased in the heart tissue and oxidative stress developed, leading to heart damage. In different studies conducted on the subject, it was determined that AZM caused heart damage, and it is stated that one of the most important mechanisms underlying this damage was oxidative stress due to ROS production.[9,28] We found that co-administration of CHR with AZM improved oxidative stress levels in cardiac tissue. Akaras et al.[16] stated that CHR is a powerful antioxidant that, reduces tissue damage, and shows this effect by suppressing ROS production and related oxidative stress. It has been reported that CHR is effective in reducing damage in different toxicity models and different tissues, suppresses ROS production, and strengthens the

antioxidant defense system.[12,17,49]

Histopathological evaluation revealed that eosinophilic changes and pyknotic nuclei in the cytoplasm of cardiomyocytes, increased vascular congestion and leukocyte infiltration confirmed the biochemical changes in AZM-induced cardiac injury. Co-administration of CHR with AZM alleviated the histopathological changes with a more regular arrangement of cardiomyocytes and less space between muscle fibers, indicating the ability of CHR to maintain myocardial structure and integrity. Saleh et al.[50] reported that CHR significantly improved cardiac sections in heart tissue against different toxic agent, with only mild myocardial blood vessel occlusion observed.

In conclusion, AZM caused cardiac marker changes, oxidative stress, tissue structural and structural changes by interfering with the damage pathways in the heart tissue. CHR, a natural flavone, showed healing effects against AZM-induced heart damage. These effects suggest that CHR can be used as a potential therapeutic agent.

Ethics Committee Approval: Ethical approval was obtained from the Atatürk University Animal Experiments Local Ethics Committee (Date: 31.01.2025, Approval No: 2025/01/22).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception- S.A., N.A., Ş.M.; Design: S.A., E.D., N.A., Ş.M.; Supervision- S.A., E.D., N.A., Ş.M.; Fundings- S.A., E.D., N.A., Ş.M.; Materials- S.A., E.D., N.A., Ş.M.; Data Collection and/or Processing- S.A., E.D., N.A.; Analysis and/or Interpretation- S.A., E.D., N.A., Ş.M.; Literature Search S.A., E.D., N.A., Ş.M.; Writing- S.A., E.D., N.A., Ş.M.; Critical Review- S.A., E.D., N.A., Ş.M.

Declaration of Interests: The authors declare no conflict of interest.

Funding: For this study, the authors did not receive any funding.

Etik Komite Onayı: Atatürk Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'ndan etik onay alındı (Tarih: 31.01.2025, Onay No: 2025/01/22).

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Tasarım: S.A., E.D., N.A., Ş.M.; Denetleme: S.A., E.D., N.A., Ş.M.; Finansman: S.A., E.D., N.A., Ş.M.; Malzemeler: S.A., E.D., N.A., Ş.M.; Veri Toplanması ve/veya İşleme: S.A., E.D., N.A.; Analiz ve/veya Yorum: S.A., E.D., N.A., Ş.M.; Literatür Taraması: S.A., E.D., N.A., Ş.M.; Yazım: S.A., E.D., N.A., Ş.M.; Eleştirel İnceleme: S.A., E.D., N.A., Ş.M.

Çıkar Çatışması: Yazarlar herhangi bir çıkar çatışması beyan etmemektedir.

Finansal Destek: Bu çalışma için yazarlar herhangi bir fon almadılar.

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ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1701588

Investigation of the Effects of Cerium Nitrate and Platelet-Rich Plasma Treatments on Rat Testicular Tissue in the Recovery of the Stasis Zone

Seryum Nitrat ve Trombositten Zengin Plazma Tedavilerinin Yanıklardan Sonra Staz Bölgesinin İyileşmesinde Sıçan Testis Dokusu Üzerindeki Etkilerinin Araştırılması

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ABSTRACT

Post-burn trauma is common in daily life. Although a burn injury causes traumatic damage to the skin, it can also cause damage to many tissues and organs, including testicular tissue, as a result of systemic inflammatory reactions in the body. Cerium nitrate (CN) and Platelet-Rich-Plasma (PRP) are natural compounds having anti-inflammatory, anti-oxidant and anti-apoptotic properties. In this study, we aimed to investigate the efficacy of CN and PRP against the damage to testicular tissues after burns. Thermal damage was induced in the skin tissues of the rats on the first day of the study. Then, the rats in the CN group were kept in a 30-min bath of 0.04 M CN. The rats in the PRP group received 0.1 ml of PRP intradermal injections in the wound area. Spermatological examinations performed after burn revealed that abnormally shaped sperm counts increased and the integrity of the sperm membrane was impaired. In histopathological examinations, thinning of the tubular walls in testicular tissues was observed, as well as a decrease in spermatocyte numbers and severe degeneration and necrosis in the spermatocytes. It was also observed that the burn triggered inflammation in testicular tissues by increasing IL-1β, TNF-α and iNOS levels, caused DNA damage by increasing 8-OHdG levels, and furthermore caused apoptosis due to increased Caspase 3 expression in the testicular cells. It was determined that CN and PRP treatments reduced the number of abnormally shaped sperms after burn, maintained membrane integrity of sperms, and suppressed inflammation, oxidative stress and apoptosis in testicular tissues.

Keywords: Apoptosis, burn, cerium nitrate, inflammation, platelet-rich plasma

ÖZ

Yanık sonrası travma günlük yaşamda sıklıkla görülmektedir. Yanık yaralanması ciltte travmatik hasara neden olmakla birlikte, vücutta sistemik inflamatuar reaksiyonlar sonucu testis dokusu da dahil olmak üzere birçok doku ve organda da hasara neden olabilir. Seryum nitrat (SN) ve Trombositten Zengin Plazma (TZP), antiinflamatuar, antioksidan ve antiapoptotik özelliklere sahip doğal bileşiklerdir. Çalışmanın birinci gününde sıçanların cilt dokularında termal hasar oluşturuldu. Daha sonra CN grubundaki sıçanlar 0,04 M SN'li 30 dakikalık banyoda tutuldu. PRP grubundaki sıçanlara yara bölgesine 0,1 ml PRP intradermal enjeksiyonu yapıldı. Yanık sonrası yapılan spermatolojik incelemelerde anormal şekilli sperm sayısının arttığı ve sperm zarının bütünlüğünün bozulduğu görüldü. Histopatolojik incelemelerde testis dokularında tubüler duvarlarda incelme, spermatosit sayısında azalma ve spermatositlerde ileri derecede dejenerasyon ve nekroz gözlendi. Yanığın testis dokularında IL-1β, TNF-α ve iNOS düzeylerini artırarak inflamasyonu tetiklediği, 8-OHdG düzeylerini artırarak DNA hasarına neden olduğu ve ayrıca testis hücrelerinde Kaspaz 3 ekspresyonunun artmasına bağlı olarak apoptoza neden olduğu gözlendi. CN ve PRP tedavilerinin yanık sonrası anormal şekilli sperm sayısını azalttığı, spermlerin membran bütünlüğünü koruduğu, testis dokularında inflamasyonu, oksidatif stresi ve apoptozu baskıladığı belirlenmiştir.

Anahtar Kelimeler: Apoptozis, inflamasyon, seryum nitrat, trombositten zengin plazma, yanık

Received/Geliş Tarihi: 18.05.2025
Accepted/Kabul Tarihi: 01.08.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Bolat İ, Kapakin KAT, Ömür AD, Özer F, Kirman EM. Investigation of the effects of cerium nitrate and platelet-rich plasma treatments on rat testicular tissue in the recovery of the stasis zone. *Vet Sci Pract*. 2025;20(2):78-86.

Atıf: Bolat İ, Kapakin KAT, Ömür AD, Özer F, Kirman EM. Seryum nitrat ve trombositten zengin plazma tedavilerinin yanıklardan sonra staz bölgesinin iyileşmesinde sıçan testis dokusu üzerindeki etkilerinin araştırılması. *Vet Sci Pract*. 2025;20(2):78-86.

INTRODUCTION

Burn injury is one of the most serious types of trauma that people are likely to experience at least once in their lifetime. At the present time, with the development of industrialization, many forms of trauma, particularly burns, are common in our daily lives. Burns usually occur by contact with a burning or hot material. As a result of the burn occurring in the tissues, due to the energy emission from the burning substance, coagulation necrosis develops within tissues and organs. The severity of the burn injury varies by the dose and severity of the flammable or combustible material, contact time of the tissue with the burning material, and the resistance of the exposed tissue.[1,2] Multisystem involvement of various tissues and organs, especially cardiovascular, respiratory and urinary systems, can be seen in very severe burns.[2] This is caused by oxidative stress and inflammatory reactions that develop in tissues and organs after burns.[3] Proinflammatory mediators cause severe inflammation and edema in the skin due to inflammatory reactions after burns. In addition to that, the inflammatory response that develops after a severe burn transforms into a systemic inflammatory response (SIRS3), affecting many tissues and organs.[4,5] Oxidative stress can cause permanent damage in the protein structures of cells, cell membranes, and lipid structure of cell DNA,[6] and at the same time, apoptosis can be observed in many cells.[7]

Although many tissues and organs in the body are severely affected by complications following burns, testicular tissues are affected even more because they are more sensitive to temperature changes. Testes are located in the scrotum sac at a temperature several degrees below the normal body temperature, which is very sensitive for spermatogenesis in testicular tissue. Oxidative stress and inflammatory reactions, which will occur due to the factors causing an increase in body temperature, such as burns, will also increase the temperature of the testes, leading to a serious decline in fertility and development of many diseases and damage, particularly cryptorchidism.[8-10] Burn wound treatment is still the subject of many experimental studies. Different treatment methods such as stem cells are widely used in studies, besides anticoagulants, anti-inflammatory, anti-thrombotic and anti-oxidant drugs. Essential active substances such as cerium nitrate (CN) and Platelet Rich Plasma (PRP) are used in these treatment methods.[5] CN, found very rare in nature, was determined to eliminate the immunosuppressive effect in the body after trauma, and to support the treatment positively by suppressing the inflammatory reaction after burn injury.[3] It was proven that PRP, obtained from the platelet-enriched plasma portion after centrifugation of blood, accelerates wound healing by affecting the release of growth hormones and anti-inflammatory cytokines in the body.[8] Nonetheless, PRP has been widely used in many treatment methods in recent years, especially in surgery and dermatology. Recent studies also revealed that PRP was very effective in the treatment of burn wounds.[10]

Various methods were used in this study, with a view to determine whether CN and PRP had a healing effect on the damage occurring in the testicular tissue after burns.

MATERIALS AND METHODS

Experimental Animals

Experimental animals to be used in the study were obtained from Atatürk University Medical and Experimental Application and Research Center (ATADEM), and the experimental process was carried out in the ATADEM. Eighty Sprague Dawley male rats were used in the study, each weighing 250-300 g and 12 weeks old. Prior to the experiment, the rats were housed at room temperature (25 ^{o}C) for 7 days, under appropriate conditions, given only feed and water in order to get adapted to the experimental environment. Eighty rats were randomly divided into 4 groups of 20. This study was carried out with the permission of Atatürk University Animal Experiments Local Ethics Committee (Date: 31.08.2021, No: 2021/194).

Establishing Burn Model in RatsIn the study, thermal damage was produced on the skin of the animals, by applying the Burn Comb Model. This was applied symmetrically without pressure on the back of the rats for 20 seconds after the special brass comb 1x2 cm in size, having 4 strings and 0.5x1 cm spaces was kept in 100 °C boiling water for 5 minutes.

Preparation of PRP

In the control group without burn, 1ml blood sample was collected into tubes containing 0.5 ml sodium citrate and centrifuged in two stages. First at 1700 rpm for 15 minutes, to seperate the red blood cell and plasma fractions, then at 3000 rpm for 5 minutes, to remove the platelet-poor plasma, and finally PRP was obtained.

Experimental Groups

Control Group: Rats were kept in a pool filled with physiological saline for 30 minutes.

Sham Group: Dermal damage was created on the skin of the rats the first day, and they were kept in 0.9% saline solution for 30 minutes.[11]

Cerium Nitrate (CN) Group: Rats were kept in bath containing 0.04 M CN for 30 minutes.

Platelet-Rich Plasma (PRP) Group: 0.1 ml of PRP was injected intradermally into the wound area of the rats with thermal damage.[12]

Semen Evaluation

One of cauda epididymidis was used to obtain semen sample for each animal. For this purpose, randomly selected cauda epididymidis was minced in Petri dish including 5 mL of physiological saline. To provide the migrations of spermatozoa from cauda epididymidis to fluid, the solution-tissue mixture was incubated in a warmed stage at 35 °C for 5 min. Following the incubation period, cauda epididymidis residue was removed by using anatomical tweezers from the Petri dish. The fluid remaining in the Petri dish was used as semen sample. Evaluation of semen was conducted using routine spermatological parameters including dead sperm rate and morphological examination of spermatozoa.

To determine the percentage of morphological abnormality of spermatozoa, the method (with a little modification by using only eosin dye instead of eosinnigrosin dye) described by Turk et al.[13] was used. Briefly, two slides for each semen sample were stained with eosin dye. Then, the slides were evaluated under light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan). Two hundred spermatozoa from each slide were examined and the numbers of spermatozoa with abnormal head were expressed as percentage.

Sperm viability was evaluated with light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan) after eosin nigrosin staining.[13] The smear was prepared for counting. A total of 200 cells were counted and the results are presented as percentages.

Taking Tissue Samples

Three weeks after burn damage, 10 rats randomly selected from each group were sacrificed under general anesthesia (Xylazine + Ketamine) and testicular tissue samples were obtained for fertility, sperm motility, histopathological and immunohistochemical examinations.

Histopathological Examinations

All testicular samples were fixed in 10% buffered formaldehyde. Tissue blocks were prepared after routine tissue follow-up steps and 4 µm thick sections were taken from the blocks. The slides were stained with Hematoxylin-Eosin (HE) and examined under a light microscope (Olympus BX51, Japan). Sections were evaluated as absent (-), mild (+), moderate (++), and severe (+++) according to histopathological findings.[14,15]

Immunohistochemical Examinations

In the immunohistochemical staining procedure, the primary antibody used was (IL-1β cat no: sc-52012, diluent ratio: 1/200 US; TNF-α cat no: sc-52746, diluent ratio: 1/200 US; iNOS: cat no: sc-7271, diluent ratio: 1/200 US) and it was incubated with the instructions for use. HRP/DAB (3,3-diaminobenzidine) was used as the chromogen, and Mayer's hematoxylin solution was used for background staining. After the staining procedure, the sections examined with a light microscope (Olympus BX51, Japan). Immunohistochemical analysis staining data were evaluated in the ImageJ analysis program.[16]

Immunofluorescence Examinations

In the immunofluorescence staining procedure, the primary antibody (8-OHdG cat no: sc-66036, diluent ratio:1/200 US; Caspase 3 cat no: 56036, diluent ratio:1/200, US) was used and incubated in accordance with the instructions for use. As secondary antibody, FITC solution (cat no: ab6717 dliuent ratio:1/500 UK) was applied to the tissues according to the instructions for use. After the staining procedure, the sections examined by fluorescence microscopy (ZEISS AXIO, Germany). Immunofluorescence analysis staining data were evaluated in the ImageJ analysis program.[17,18]

Statistical Analysis

The statistical analysis for histopathological examinations was performed using GraphPad Prisim program, with $P < .05$ considered as significant in data evaluation. Mann-Whitney U test was used for determining differences between groups. For the statistical analysis of spermatological parameters, parametric RM-one-way ANOVA was performed, followed by Tukey's tests.

RESULTS

Spermatological Examination Findings

Spermatological examinations after burns were performed, and abnormal sperm counts and membrane damage were determined and presented in Table 1.

Table 1. Evaluation of study groups in terms of spermatological parameters (x±SEM)

Group	Total abnormal sperm cell %	Membrane integrity %
Control	15.50±3.39 [a]	34.50±7.31[a]
Sham	23.50±4.92[b]	25.00±4.47[b]
CN	22.83±2.48[b]	29.50±2.34[ab]
PRP	16.66±5.16[a]	32.50±5.24[a]
p	*	*

a,b: Differences between means with different letters in the same column are significant (*: $P < .05$), CN: Cerium nitrate, PRP: Platelet-Rich-Plasma

Hematoxylin Eosin Staining Findings

Control Group: Testicular tissues displayed a normal histological structure (Figure 1A).

Sham Group: Severe degeneration and necrotic changes were noted, along with considerable thinning of the walls of seminiferous tubules, and a significant decrease in the number of Sertoli cells and spermatocytes of the testicular tissues. At the same time, significant decrease in the number of spermatozoa in the tubulus lumens and intense hyperemia in the intertubular veins and a slight lymphocyte cell infiltration were observed (Figure 1B).

CN Group: Mild thinning of the walls of the seminiferous tubules, moderate degeneration with a decrease in the number of Sertoli cells and spermatocytes were noted. At the same time, a decrease in the number of spermatozoa in the tubular lumens and moderate hyperemia in the veins in the intertubular areas were observed (Figure 1C).

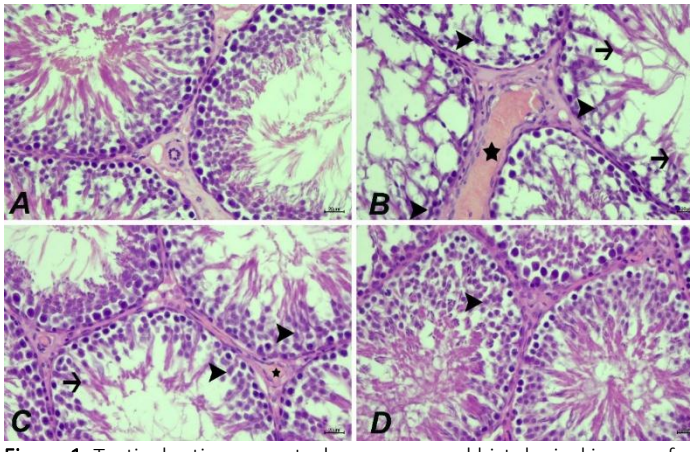

Figure 1. Testicular tissue, control group; normal histological image of testicular tissue (A), sham group; severe degeneration of spermatocytes (arrowheads), moderate necrosis of spermatocytes (arrows), severe hyperemia (star) of veins (B), CN group; moderate degeneration of spermatocytes (arrowheads), mild necrosis of spermatocytes (arrows), moderate hyperemia (star) in veins (C), PRP group; mild degeneration of spermatocytes (arrowheads) (D), H&E, Bar: 20µm

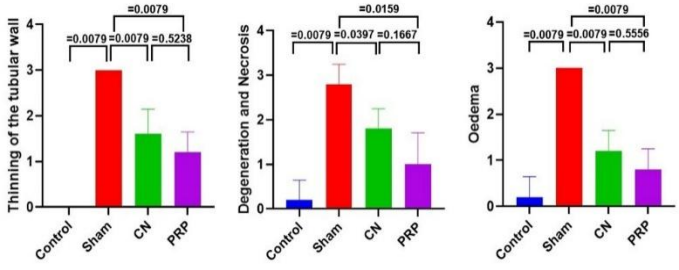

Figure 2. Scoring of histopathological findings seen in testicular tissue and statistical analysis data. Mann-Whitney U test was used for comparison between groups.

PRP Group: Mild degeneration in testicular tissue with mild decrease in Sertoli cells and spermatocyte count and moderate hyperemia in intertubular vein areas were determined (Figure 1D). Histopathological findings and scoring are presented in Figure 2.

Immunohistochemical Examination Findings

Control Group: Immunohistochemical staining of testicular tissues showed that IL-1β, TNF-α and iNOS expressions were negative (Figure 3A,4A,5A).

Sham Group: As a result of immunohistochemical staining, IL-1β, TNF-α and iNOS expressions were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells. In this group, IL-1β expression was moderate, while TNF-α and iNOS expressions were significantly more severe than IL-1β expression (Figure 3B,4B,5B).

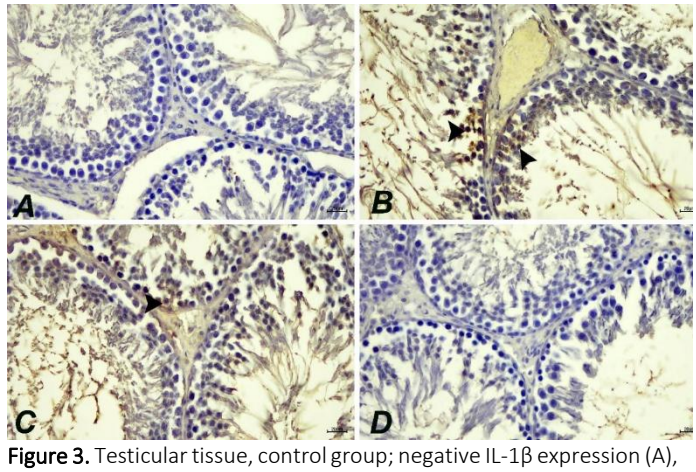

Figure 3. Testicular tissue, control group; negative IL-1β expression (A), sham group; moderate IL-1β expression (arrowheads) (B), CN group; mild IL-1β expression (arrowheads) (C), PRP group; negative IL-1β expression (D), IHC, Bar:20µm

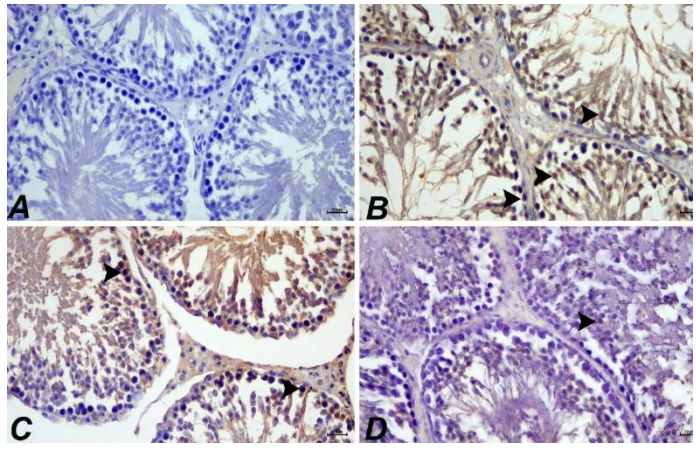

Figure 4. Testicular tissue, control group; negative TNF-α expression (A), sham group; severe TNF-α expression (arrowheads), CN group; moderate TNF-α expression (arrowheads), PRP group; slight expression of TNF-α (arrowheads), IHC, Bar:20µm

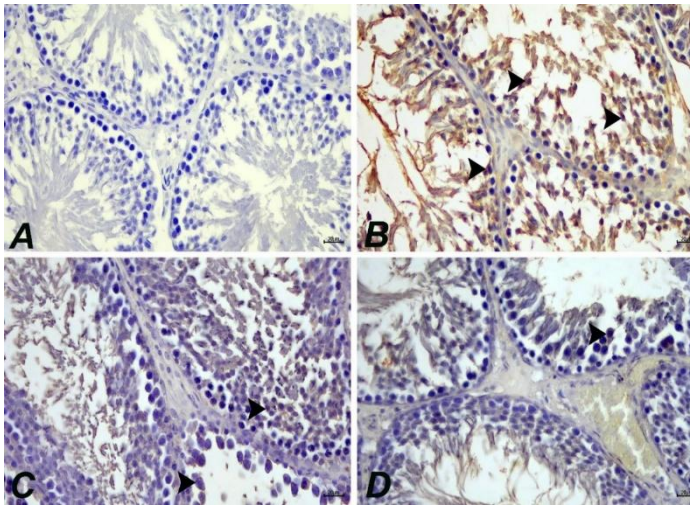

Figure 5. Testicular tissue, control group; negative iNOS expression (A), sham group; severe iNOS expression (arrowheads) (B), CN group; moderate iNOS expression (arrowheads) (C), PRP group; mild iNOS expression (arrowheads) (D), IHC, Bar: 20µm

CN Group: IL-1β, TNF-α and iNOS expressions were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells. In this group, IL-1β expression was mild, while TNF-α and iNOS expressions were severe with respect to IL-1β expression (Figure 3C, 4C, 5C).

RP Group: TNF-α and iNOS expressions were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells, while IL-1β expression was not observed (Figure 3D, 4D, 5D). Immunohistochemical findings scoring are presented in Figure 6.

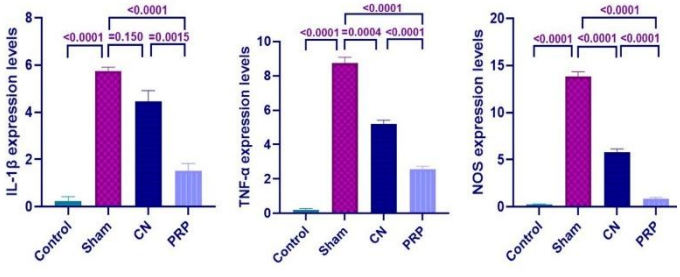

Figure 6. Immunohistochemical analysis results and statistical analysis data calculated in the ImageJ analysis program on testicular tissue. One-way ANOVA followed by Tukey's test was performed for statistical evaluation of the data in the study.

Immunofluorescence Examination Findings

Control Group: Immunofluorescence staining of testicular tissues revealed that 8-OHdG and Caspase 3 expressions were negative (Figure 7A, 8A).

Sham Goup: 8-OHdG and Caspase 3 expressions were found to be severe in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells of testicular tissues (Figure 7B, 8B).

CN Group: Moderate levels of expression of 8-OHdG and Caspase 3 were detected in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells (Figure 7C, 8C).

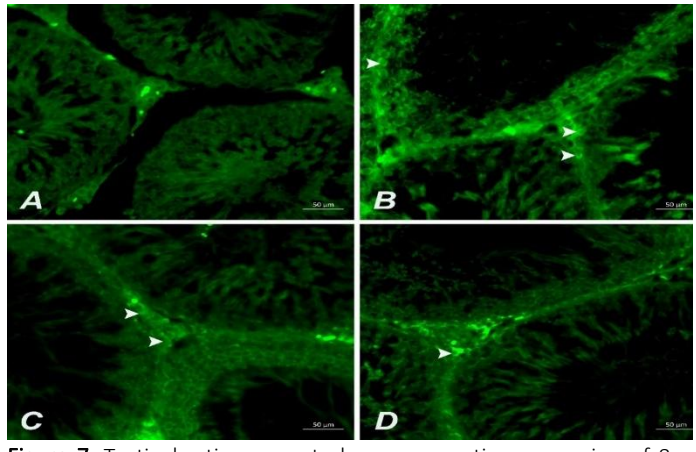

Figure 7. Testicular tissue, control group; negative expression of 8-OHdG (A), sham group; severe 8-OHdG expression (arrowheads) (B), CN group; moderate expression of 8-OHdG (arrowheads) (C), PRP group; slight expression of 8-OHdG (arrowheads) (D), IF, Bar: 50µm

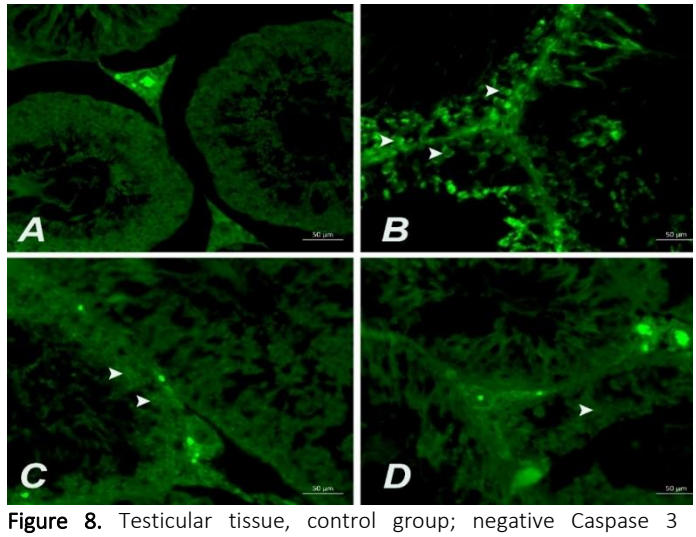

Figure 8. Testicular tissue, control group; negative Caspase 3 expression (A), sham group; severe Caspase 3 expression (arrowheads) (B), CN group; moderate Caspase 3 expression (arrowheads) (C), PRP group; light Caspase 3 expression (arrowheads) (D), IF, Bar: 50µm

PRP Group: 8-OHdG and Caspase 3 expressions were found to be mild in leydig, sertoli and germ (spermatocytes, spermatogonia and spermatid) cells of testicular tissues (Figure 7D, 8D). Immunofluorescence findings scoring are presented in Figure 9.

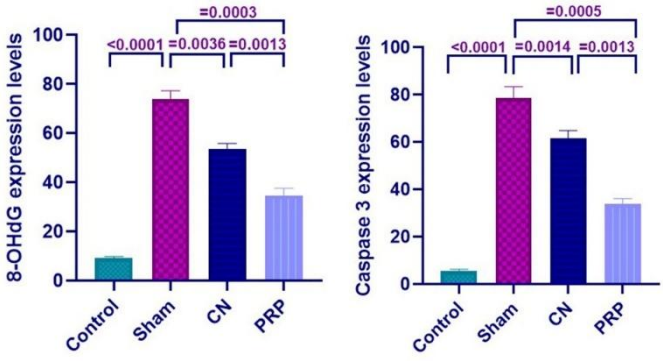

Figure 9. Immunofluorescence analysis results calculated in the ImageJ analysis program in testicular tissue and statistical analysis data. In the study, one-way ANOVA and then Tukey's test were performed for statistical evaluation of the data.

DISCUSSION

As a result of industrialization, burn is one of the the most common type of trauma today.[1,2] The term "burn" immediately reminds of dermal injury.[19,20] However, it was reported that vital organs such as liver[21] and kidney,[22] as well as testicular tissue,[23] are affected very severely due to increased body temperature in burns.

There are many studies in the literature today, using different active substances for the treatment of burn wounds.[24,25] Cerium nitrate is one of these substances, and widely used for a long time. CN was first used in burn treatment in a study conducted in 1976, demonstrating its healing properties.[26] After that, it started to be widely used in the treatment of burns.[27] PRP is another substance that was shown to be effective in the treatment of burns in recent years, though its history is not as old as CN. Many studies demonstrated its effectiveness in the treatment of burn damages.[28] In the present study where a skin burn wound was induced, it was demonstrated for the first time by histopathological, immunohistochemical, immunofluorescent and spermatological examinations that testicular tissue damage occurred due to burn and CN and PRP had a protective effect against this damage.

In studies on burns, testicular damage and impaired spermatogenesis have been observed.[23] It is seen that testicular damage findings obtained from burn studies support the spermatological findings in our study. Besides, there are studies in which many agents are used to determine the protective efficacy on testicular damage in rats.[29,30] In a study in rats, it was observed that PRP (80µl, testis local injected, single dose) application had a protective effect on spermatological parameters.[31] Similarly, the positive effects of Cerium Nitrate (CN) and Platelet-Rich Plasma (PRP) application, which we used in our study, on testicular tissue were observed.

Testicular damage and impaired spermatogenesis have been observed in studies on burns.[23] The results of these studies, indicating testicular damage, support the spermatological findings obtained in our study. There are also studies examining the protective effects of various agents on testicular damage in rats.[29,30] In a study with rats, it was observed that PRP application (80µl, intratesticular injection, single dose) had a protective effect on spermatological parameters.[31] Similarly, CN and PRP applications in our study were observed to have positive effects on testicular tissue.

A burn on the skin causes damage not only to the skin tissue, but also various tissues and organs are seriously affected, particularly the testicular tissue which is very sensitive to body temperature changes.[23] Severe damage was determined in the testicular tissues and in the seminiferous tubules of the rats induced with severe burn trauma as well as a significant reduction was observed in the number of cells responsible for sperm production and the number of mature sperm cells in the lumen.[23] In the present study too, damage to the seminiferous tubules, degeneration and necrosis of the spermatocytes were observed, indicating that the burn actually affected not only the skin tissue but also the testicular tissue at a very severe level. Although CN[32,33] and PRP[34] are widely used in the treatment of skin burns, their effects on testicular tissue have not been revealed yet in the literature. This study determined that CN and PRP treatments can be effective against histopathological lesions in testis tissue.

A severe burn trauma may cause inflammatory reactions in various tissues and organs due to the development of a systemic inflammatory response (SIRS3) in the body, although mild burn traumas generally cause local inflammatory responses.[4,5] Testicular tissue is the best example in this respect. It was reported that the inflammatory response develops very quickly after the burn due to the thermal sensitivity of the testicular tissue and its vulnerability against heat.[35] Although it is well known that a systemic inflammatory response develops in the body after a very severe burn, still the studies on testicular tissue remained very limited. A study conducted in this context reported that serum TNF alpha levels increased very severely after burns in rats.[35] It was determined in the present study, that the expression levels of IL-1β, TNF-α and iNOS increased very severely in testicular tissues due to systemic inflammatory reactions. This study revealed that the testicular tissue can also be severely affected by the systemic inflammatory reaction developing after the burn.

Today, there are many anti-inflammatory components such as CN[27,36] and PRP[37] for suppressing the systemic

inflammatory response of the body. To the best of our knowledge, there is no study in the literature investigating the anti-inflammatory effects of both CN and PRP on testicular tissues after burns, although this was demonstrated in many studies on trauma[38] and diseases.[37] This study revealed that CN and PRP have anti-inflammatory effects on testicular tissues against systemic inflammatory reactions occurring after burns.

It has been reported in many studies that free radicals released due to thermal skin damage after burns, increase oxidative stress in tissues causing DNA damage and subsequently leading cells to apoptosis depending on the severity of DNA damage.[39] In this study, where a burn wound was created/induced on the skin, increased levels of 8-OHdG expressions observed in spermatocytes indicated that testicular tissue was highly affected by oxidative stress.[40] Many studies demonstrated that both CN[41] and PRP[42,43] suppressed oxidative stress in the body. This study determined that CN and PRP active substances used in treatments, significantly reduced both 8-OHdG and Caspase 3 expression levels in spermatocytes by suppressing oxidative stress.

As a result, it was determined that the systemic inflammatory response and oxidative stress that occur in the body after burns cause inflammation in testicular tissues, as well as DNA damage and apoptosis in spermatocytes, and that CN and PRP have a protective effect against these damages. It is thought that the protective effects of CN and PRP occur by suppressing the systemic inflammatory reactions and oxidative stress that occur after burns.

Ethics Committee Approval: This study was carried out with the permission of Atatürk University Animal Experiments Local Ethics Committee (Date: 31.08.2021, Decision No: 2021/194).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – İ.B., K.A.T.K.; Design- İ.B., K.A.T.K.; Supervision- İ.B. K.A.T.K; Resources- İ.B. E.M.K., K.A.T.K.; Data Collection and/or Processing- İ.B., A.D., F.Ö., K.A.T.K.; Analysis and/or Interpretation-K.A.T.K.; Literature Search- İ.B., E.M.K.; Writing Manuscript- İ.B., E.M.K.; Critical Review- İ.B., K.A.T.K.

Declaration of Interests: The authors declare that there is no conflict of interest.

Funding: The authors declared that they received no financial support for this study.

Etik Komite Onayı: Bu çalışma Atatürk Üniversitesi Hayvan Deneyleri Yerel Etik Kurulunun izni ile yürütülmüştür (Tarih: 31.08.2021, Karar Numarası: 2021/194).

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Fikir - İ.B., K.A.T.K.; Tasarım - İ.B., K.A.T.K.; Denetleme - İ.B. K.A.T.K; Kaynaklar - İ.B. E.M.K., K.A.T.K.; Veri Toplanması ve/veya İşlemesi - İ.B., A.D., F.Ö., K.A.T.K.; Analiz ve/ veya Yorum – K.A.T.K.; Literatür Taraması – İ.B., E.M.K.; Yazıyı Yazan – İ.B., E.M.K.; Eleştirel İnceleme – İ.B., K.A.T.K.

Çıkar Çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

Finansal Destek: Yazarlar bu çalışma için herhangi bir mali destek almadıklarını beyan etmişlerdir.

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Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci.1708883

Molecular Detection and Partial Membrane Gene Sequence-Based Characterization of Canine Coronavirus in Diarrheal Shelter Dogs in Sivas, Türkiye

Türkiye, Sivas'taki İshalli Barınak Köpeklerinde Köpek Koronavirüsünün Moleküler Tespiti ve Kısmi Membran Gen Dizisine Dayalı Karakterizasyonu

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ABSTRACT

Canine coronavirus (CCoV) infection is widespread among dogs worldwide and is a known cause of gastroenteritis. It is particularly lethal in puppy populations. The virus spreads easily in environments where dogs are densely populated, such as animal shelters, and is shed through feces. This study aimed to detect the presence and molecular characteristics of canine coronavirus (CCoV) in shelter dogs with diarrhea at the Sivas Municipality Animal Shelter. Fecal samples were collected from 150 shelter dogs (127 adults and 23 puppies) showing diarrheal symptoms. RT-PCR analysis was performed using two primer sets targeting the CCoV M gene (CCV1/CCV2 and CCV1a/CCV2). The CCV1/CCV2 primer pair showed that 38% (57/150) of diarrheic dogs tested positive for CCoV. The positivity rate was 31.50% (40/127) in adult dogs and 73.91% (17/23) in puppies. Typing was performed using the second primer set (CCV1a/CCV2) based on the M gene. Of the 57 positive samples, 6 (10.53%) were CCoV Type II and 51 (89.47%) were CCoV Type I. RT-PCR sequence analysis, bioinformatics evaluation, and phylogenetic assessment were conducted. These findings indicate that CCoV Type I was responsible for the majority of coronavirus infections causing diarrhea in the studied dog population.

Keywords: Diarrhea, dog coronavirus, M gene, phylogenetic analysis

ÖZ

Köpek koronavirüsü (CCoV) enfeksiyonu dünya çapında köpekler arasında yaygındır ve gastroenteritin bilinen bir nedenidir. Özellikle yavru köpek popülasyonlarında öldürücüdür. Virüs, hayvan barınakları gibi köpek yoğunluğunun yüksek olduğu ortamlarda kolayca yayılır ve dışkı yoluyla yayılır. Bu çalışma, Sivas Belediye Hayvan Barınağı'ndaki ishalli barınak köpeklerinde köpek koronavirüsünün (CCoV) varlığını ve moleküler özelliklerini tespit etmeyi amaçlamıştır. İshal semptomları gösteren 150 barınak köpeğinden (127 yetişkin ve 23 yavru) dışkı örnekleri toplandı. CCoV M genini (CCV1/CCV2 ve CCV1a/CCV2) hedef alan iki primer seti kullanılarak RT-PCR analizi gerçekleştirildi. CCV1/CCV2 primer çifti, ishalli köpeklerin %38'inin (57/150) CCoV için pozitif olduğunu gösterdi. Pozitiflik oranı yetişkin köpeklerde %31,50 (40/127) ve yavru köpeklerde %73,91 (17/23) idi. Tiplendirme, M genine dayalı ikinci primer seti (CCV1a/CCV2) kullanılarak gerçekleştirildi. 57 pozitif örnekten 6'sı (%10,53) CCoV Tip II ve 51'i (%89,47) CCoV Tip I olarak belirlendi. RT-PCR dizi analizi, biyoenformatik değerlendirme ve filogenetik değerlendirme yapıldı. Bu bulgular, CCoV Tip I'in incelenen köpek popülasyonunda ishale neden olan koronavirüs enfeksiyonlarının çoğundan sorumlu olduğunu göstermektedir.

Anahtar Kelimeler: Filogenetik analiz, ishal, köpek coronavirus, M gen

Received/Geliş Tarihi: 30.05.2025
Accepted/Kabul Tarihi: 01.08.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Turan T, Işıdan H, Atasoy MO. Molecular detection and partial membrane gene sequence-based characterization of canine coronavirus in diarrheal shelter dogs in Sivas, Türkiye. *Vet Sci Pract*. 2025;20(2):87-93.

Atıf: Turan T, Işıdan H, Atasoy MO. Türkiye, Sivas'taki ishalli barınak köpeklerinde köpek koronavirüsünün moleküler tespiti ve kısmi membran gen dizisine dayalı karakterizasyonu. *Vet Sci Pract*. 2025;20(2):87-93.

INTRODUCTION

The global dog population is estimated at approximately 700 million, of which around 300 million are stray dogs. This corresponds to roughly one dog for every ten people.[1] It is widely accepted that the dog was the first domesticated animal, originating from the domestication of wolves in the Middle East approximately 12,000 years ago.[2] Gastroenteritis is one of the most common health problems affecting dogs.[3-5]

Coronaviruses (order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*) are enveloped, positive-sense single-stranded RNA viruses with the largest known RNA genomes, ranging from 27 to 31 kb. The *Orthocoronavirinae* subfamily comprises four genera: *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus*, and *Gammacoronavirus*. CCoV belongs to the *Tegacovirus* subgenus within the *Alphacoronavirus* genus, alongside feline coronavirus (FCoV) and transmissible gastroenteritis virus of pigs (TGEV).[6] The CCoV genome contains four open reading frames (ORFs) encoding the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N), as well as several ORFs translating into non-structural proteins, including RNA-dependent RNA polymerase polyprotein precursors.[7-9] CCoV was first described in 1971 in association with diarrheal outbreaks in dogs.[10] It is widely accepted as the cause of canine gastroenteritis, which is characterized by symptoms such as anorexia, lethargy, diarrhea, and vomiting. These symptoms typically last up to two weeks. Although clinical signs are usually mild and mortality is low, the virus is highly contagious.[11, 12] It is prevalent worldwide, having been identified or isolated in Europe, the United States, Asia, and Australia.[12-17]

MATERIALS AND METHODS

Sampling

In this study, 150 rectal swab samples were collected from dogs at the Sivas Municipality Animal Shelter between 2019 and 2020. The samples comprised 127 adult dogs (over 1 year old) and 23 puppies (2–4 months old), all exhibiting symptoms of gastroenteritis and diarrhea. Following collection, specimens were transported to the laboratory and stored at −80°C prior to RNA extraction. (Ethics Committee Decision No: 65202830-050.04.04-34, Date: 15.05.2025)

RNA Isolation

Swab samples were diluted 1:10 with 1 M phosphate-buffered saline, then centrifuged at 3,500 rpm for 10 minutes to remove coarse particles. A 200 µl aliquot of the resulting supernatant was used for extraction with a commercial Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) following the manufacturer's instructions. The extracted nucleic acids were stored at −80°C until further analysis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA synthesis was performed in a total volume of 20 µl, containing 5 µl of RNA extract, 10 mM deoxynucleoside triphosphates (dNTPs), 2.5 µl of 10× RT buffer (50 mM Tris-HCl, pH 8.3 at 25°C; 75 mM KCl; 3 mM $MgCl_2$; and 10 mM DTT), 50 ng of random hexamers, 40 U of RNasin, and 200 U of M-MuLV Reverse Transcriptase RNase H (Vivantis, Germany). Reverse transcription was carried out at 37°C for 1 hour. The resulting cDNA samples were amplified using the CCV1/CCV2 and CCV1a/CCV2 primer sets described by Pratelli et al.[18] (Table 1).

Table 1. Oligonucleotide primers used for the detection and sequencing of a partial region of the M gene of CCoV

Primer name	Sequence (5'-3')	Target gene	Amplicon size
CCV1*	TCCAGATATGTAATGTTCGG	M gene of Canine coronavirus	410 bp
CCV1a*	GTGCTTCCTCTTGAAGGTACA		239 bp
CCV2*	TCTGTTGAGTAATCACCAGCT		

*Pratelli et al.[18]

PCR was performed in a final volume of 50 µl, using 5 µl of the reverse transcription (RT) reaction mixture as template. The PCR mixture contained 5 µl of 10× PCR buffer, 10 mM dNTPs, 10 pmol/µl of each forward and reverse primer, and 5 U of Taq DNA polymerase (Vivantis, Germany).

Molecular detection of the partial M gene of CCoV was conducted using the primer sets listed in Table 1. The PCR conditions were as follows: initial denaturation at 95°C for 3 minutes; 40 cycles of denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds (primer set 1) or 52°C for 30 seconds (primer set 2), and extension at 72°C for 1 minute; followed by a final extension at 72°C for 10 minutes.

Sequencing and Phylogenetic Analysis

PCR amplicons were purified using the Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced with the Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). All sequenced products were used for phylogenetic analysis. We compared partial M gene sequences with reference sequences obtained from the

National Center for Biotechnology Information (NCBI) database. Sequence alignment and phylogenetic analysis based on a 410 bp partial nucleotide sequence of the M gene were performed using Geneious Prime 2025.1.2 software.[19]

RESULTS

This molecular investigation was based on the detection of 410 bp and 239 bp amplicons specific to the M gene of the CCoV genome, which were amplified using the primer sets listed in Table 1. The CCoV results for a total of 150 dog fecal samples with diarrhea, tested with the PCR primer sets, are summarized in the table below (Table 2).

Among the 127 adult dogs, 40 fecal samples (31.50%) tested positive for the 410 bp partial M gene of CCoV. In contrast, 17 fecal samples (73.91%) from puppies were positive. The 57 samples positive with the CCV1/CCV2 primer pair were further analyzed by PCR using the CCV1a/CCV2 primer pair. Of these, 51 yielded a Type I-specific 239 bp product, while 6 samples failed to produce a positive result and were classified as Type II.

In this study, partial sequence data from 14 samples (410 bp M gene), obtained using the primer pair described by Pratelli et al.[18], were compared both among themselves and with existing sequences in GenBank. For this purpose, a phylogenetic tree was constructed using the Tamura-Nei Neighbor Joining substitution model.[20] Of these sequences, 8 (SivasCCoV-4, 20, 26, 46, 60, 67, 76, 102) were confirmed as CCoV Type I. Meanwhile, 6 sequences (SivasCCoV-3, 16, 43, 65, 78, 116), which did not yield a 239 bp product using the Type I-specific primer pair, were classified as CCoV Type II (Figure 1).

According to the consensus sequence, this mutation involved a TTT→CTC change at the first and third nucleotide positions of the codon encoding the 128th amino acid, resulting in a phenylalanine-to-leucine substitution (F→L). This change was observed in six of the eight CCoV Type I isolates: SivasCCoV-26, 46, 60, 67, 76, and 102 (MK507576–MK507581). A similar amino acid substitution was also detected in the Turkish isolate TR-Erz-B1-K34 (MN913446) (Figure 2).

Table 2. Overall PCR results for the detection of canine coronavirus in both adult dogs and puppies.

	Total Samples	Total	CCoV Type I	CCoV Type II
Adults	127	31.50% (40/127)	95.0% (38/40)	5.0% (2/40)
Puppies	23	73.91% (17/23)	76.47% (13/17)	23.53% (4/17)
Adults + Puppies	150	38.00% (57/150)	89.47% (51/57)	10.53% (6/57)

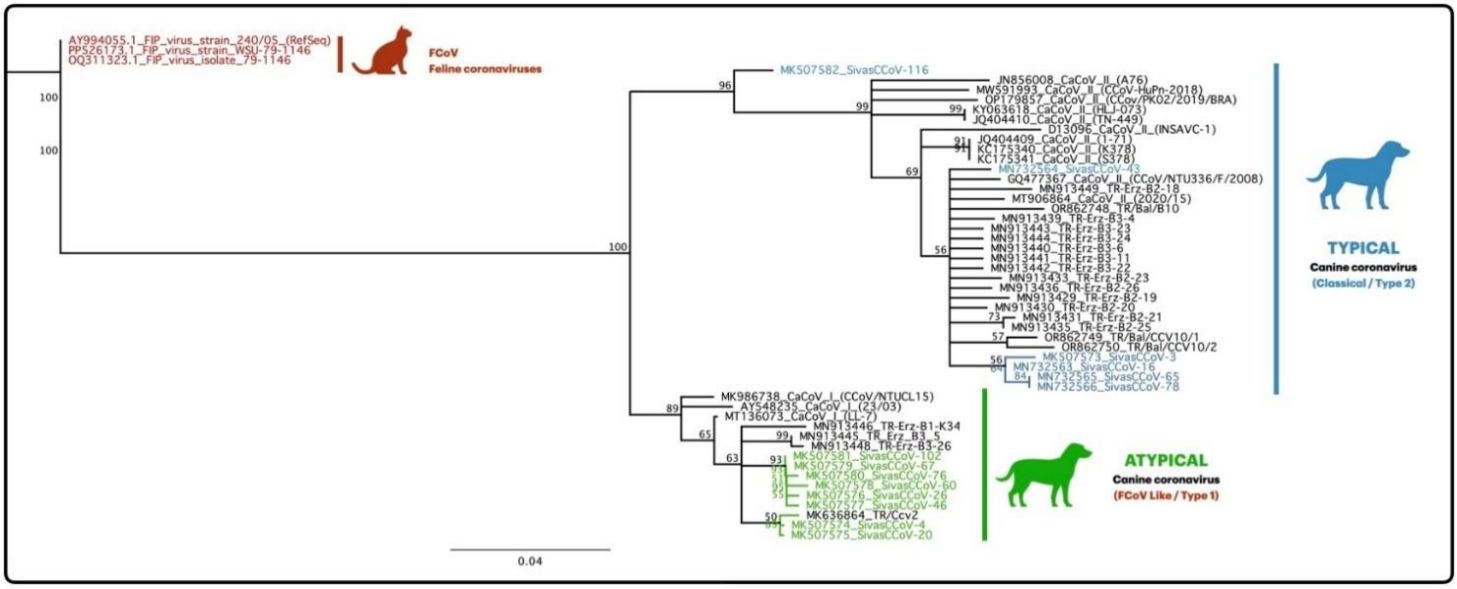

Figure 1: A consensus tree based on a 410 bp partial M gene sequence of CCoV. The phylogenetic tree was constructed using the Tamura-Nei Neighbor-Joining substitution model[20] and bootstrapped 1000 times. Feline infectious peritonitis virus (FIPV) isolates were used as an outgroup.

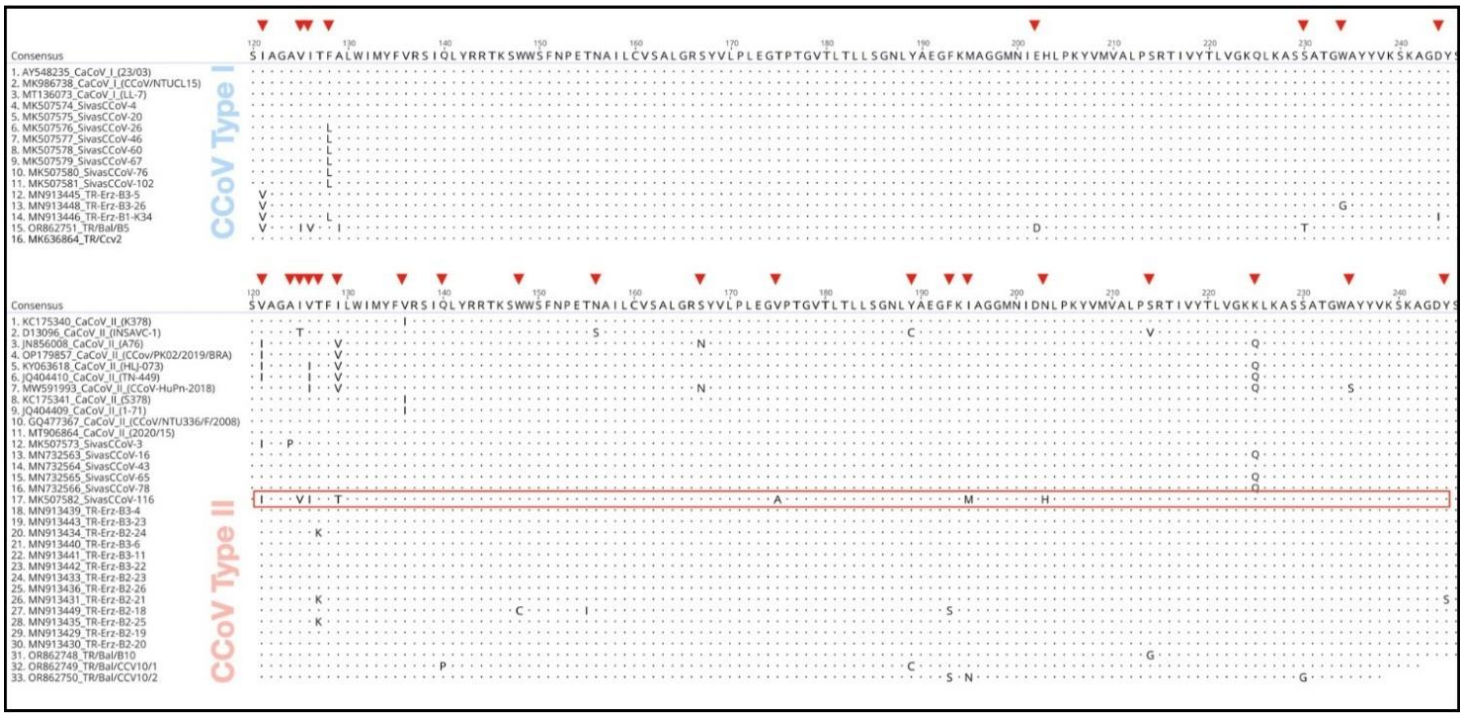

Figure 2. Amino acid differences detected in the partial M gene sequences of CCoV isolates obtained in this study, along with other sequences retrieved from the GenBank database. (–) indicates identical amino acids. Amino acids with mutations are marked with red arrows. (The SivasCCoV-116 isolate, which exhibits seven mutations, is highlighted in red.)

A significantly higher number of mutations were detected in the six isolates identified as CCoV Type II compared to the Type I isolates. The first mutation involved a GTT→ATC substitution at the first and third nucleotide positions of the codon encoding amino acid 121, resulting in a valine-to-isoleucine change (V→I). This mutation was observed in two of the six Type II isolates, SivasCCoV-3 and 116 (MK507573 and MK507582). A similar amino acid substitution was also detected in the US isolates A76 (JN856008) and TN-449 (JQ404410), the Chinese isolate HLJ-073 (KY063618), and the Brazilian isolate CCoV/PK02/2019/BRA (OP179857).

In the SivasCCoV-3 (MK507573) isolate, a GCA→CCA substitution at the first nucleotide position of the codon encoding the 124th amino acid resulted in an alanine-to-proline change (A→P). This mutation was unique, as it was not detected in any other isolate in study, nor in those reported from Türkiye or other countries.

In the SivasCCoV-116 (MK507582) isolate, a nucleotide substitution (ATT→GTT) at the first position of the codon encoding the 125th amino acid resulted in an isoleucine-to-valine change (I→V). This mutation was unique and was not detected in any other isolate. However, in the UK isolate INSAVC-1 (D13096), a different mutation was identified at the second nucleotide position of the same codon, leading to an isoleucine-to-threonine substitution (I→T) via an ATT→ACT change.

In the SivasCCoV-116 isolate (MK507582), a nucleotide substitution at the first position of the codon encoding amino acid 126 resulted in a valine-to-isoleucine change (V→I), corresponding to a GTT→ATT transition. Interestingly, this substitution (ATT→GTT; I→V) is the exact reverse of the mutation observed at position 125. The same mutation was also identified in the Chinese isolate HLJ-073 (KY063618), the US isolate TN-449 (JQ404410), and the Malaysian isolate CCoV-HuPn-2018 (MW591993).

In our SivasCCoV-116 (MK507582) isolate, a nucleotide substitution at the second position of the codon encoding the 129th amino acid (ATA→ACA) resulted in an isoleucine-to-threonine change (I→T). In contrast, the American isolates A76 (JN856008) and TN-449 (JQ404410), the Brazilian isolate CCoV/PK02/2019/BRA (OP179857), the Chinese isolate HLJ-073 (KY063618), and the Malaysian isolate CCoV-HuPn-2018 (MW591993) exhibited a different mutation at the first nucleotide position of the same codon, leading to an isoleucine-to-valine substitution (I→V) via an ATA→GTA change.

In the SivasCCoV-116 (MK507582) isolate, codon substitutions resulted in three unique amino acid changes: valine to alanine (V→A) at position 175 via a GTG→GCG mutation; isoleucine to methionine (I→M) at position 195 via an ATT→ATG mutation; and isoleucine to histidine (I→H) at position 203 via an ATT→CAT mutation. None of these mutations were observed in the other 48 sequences

analyzed. However, in the Turkish isolate TR/Bal/CCV10/2 (OR862350), the mutation at position 195 involved a different substitution— from isoleucine to asparagine (I→N) through an ATT→AAT change, rather than to methionine.

The final mutation identified in our CCoV Type II isolates was a lysine-to-glutamine substitution (K→Q) at amino acid position 225, resulting from an AAA→CAA mutation. This change was detected in the SivasCCoV-16, 65, and 78 isolates (MN732563, MN732565, and MN732566), as well as in the American isolates A76 (JN856008) and TN-449 (JQ404410), the Chinese isolate HLJ-073 (KY063618), and the Malaysian isolate CCoV-HuPn-2018 (MW591993) (Figure 2).

DISCUSSION

The primary objective of this study was to investigate the presence of CCoV in diarrheic cases frequently affecting shelter dogs in Sivas, Türkiye, and to assess its contribution to diarrhea in both adult dogs and puppies. Additionally, we analyzed the genetic diversity of CCoV isolates identified in Türkiye based on the M gene and examined their relationships with previously characterized strains. CCoV was detected in 38% (57/150) of diarrheic dogs. The detection rate was 31.5% (40/127) in adult dogs and increased to 73.91% (17/23) in puppies, suggesting a significant role of CCoV in puppy diarrhea. These rates were higher than those reported in studies from Türkiye, the United Kingdom, Portugal, Spain, and Japan[14, 21], but lower than those observed in Italy, Hungary, and Greece.[22, 23] Notably, the 73.91% positivity rate in puppies was considerably higher than the 15.5% reported by Yeşilbağ et al.[21] in Türkiye.

Of the limited number of studies focusing on the molecular characterization of canine coronavirus via M gene analysis in Türkiye, Type II has generally been reported as the predominant type.[21, 24–27] Nevertheless, in our study, 51 out of 57 samples (89.5%) were identified as Type I using the RT-PCR assay with primer pairs described by Pratelli et al.[18] These findings differ from most previous studies conducted in Türkiye. Moreover, such a pronounced predominance of Type I has not been reported in global studies.[22, 28–30]

The M gene region of coronaviruses is highly conserved and plays a crucial role in triggering robust immune responses. Consequently, it is often targeted in the detection of viral infections. However, despite its conserved nature, studies have demonstrated that mutations in the M gene may enable the virus to evade the host immune system.[27, 31–33]

Accordingly, RT-PCR analysis targeting the CCoV M gene was conducted in this study. This approach enabled the detection of nucleotide and amino acid variations, and the resulting CCoV sequences were compared with available GenBank data. Both CCoV Type I and Type II isolates identified in this study showed high similarity to previously characterized strains from Türkiye and other regions worldwide.

Our findings showed that the eight Type I isolates exhibited high nucleotide identity (99.2%–100%) with the Italian 23/03 (AY548235)[34], Taiwanese LL7 (MT136073), and Chinese CCoV/NTUCL15 (MK986738) strains. Notably, SivasCCoV-4 and -20 shared identical amino acid sequences, while the remaining six isolates (SivasCCoV-26, -46, -60, -67, -76, and -102) exhibited a single amino acid substitution from Phenylalanine to Leucine (F→L) at position 128. In a study by Akkutay Yoldar et al.[24] in Türkiye, the TR/Ccv2 isolate (MK636864) showed 99.2%–100% amino acid similarity with our sequences. Similarly, Timurkan et al.[25] reported 97.6%–99.2% similarity between our isolates and the Tr-Erz-B3-5 (MN913445), Tr-Erz-B3-26 (MN913448), and Tr-Erz-B1-K34 (MN913446) strains in GenBank. In another study conducted in Türkiye[27], similarity rates between our Type I isolates and the Tr/Bal/B5 isolate (OR862751) ranged from 94.5% to 95.3%.

The rate of amino acid changes was significantly higher in the six Types II CCoV isolates than in the Type I isolates. For instance, the SivasCCoV-116 isolate exhibited seven amino acid substitutions, resulting in identity rates ranging from 93.8% to 100%. These mutations occurred at positions 121 (Valine→Isoleucine, V→I), 125 (Isoleucine→Valine, I→V), 126 (Valine→Isoleucine, V→I), 195 (Isoleucine→Methionine, I→M), and 203 (Asparagine→Histidine, N→H). Despite these alterations, the isolate was still classified as Type II based on phylogenetic analysis (Figure 1). When compared with isolates from other countries, identity rates ranged from 92.2% to 100%. The lowest similarity was observed between SivasCCoV-116 and the UK isolate INSAVC-1 (D13096)[35], while the highest was between SivasCCoV-43 (MN732564) and the Taiwanese CCoV/NTU336/F/2008 (GQ477367) and UK 2020/15 (MT906864)[36] isolates. In comparisons with Turkish isolates, identity rates ranged from 92.1% to 100%. The lowest similarity was observed between SivasCCoV-116 and TR-Erz-B2-18 (MN913449), whereas the highest was between SivasCCoV-43 (MN732864) and nine Turkish isolates (MN913429, MN913430, MN913433, MN913436, MN913439–MN913443).[25] In another study conducted in Türkiye[27], identity rates between our isolates and three Turkish isolates ranged from 92.4% to 99.2%.

In conclusion, this study demonstrated the presence of both

Type I and Type II CCoV in adult and puppy dogs with diarrhea at the Sivas Municipality Animal Shelter. This finding was based on a partial molecular analysis of the conserved M gene. However, a clear predominance of CCoV Type I was observed. The isolates identified exhibited unique amino acid substitutions not previously reported in the literature. These findings enhance understanding of the genetic diversity of CCoV isolates circulating in Türkiye and have implications for future vaccine development efforts.

Ethics Committee Approval: Ethics committee approval was received from Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Date: 15.05.2025, Approval No: 65202830-050.04.04-34).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - TT; Design - TT; Supervision - TT; Resources – TT, HI; Materials – TT, HI, MOA; Data Collection and/or Processing – TT, HI, MOA; Analysis and/or Interpretation – TT, HI, MOA; Literature Search – TT, HI, MOA; Writing Manuscript – TT, MOA; Critical Review – HI, MOA; Other – TT

Declaration of Interests: The authors declare no conflict of interest.

Funding: No financial support was received for this study.

Etik Komite Onayı: Etik kurul onayı Sivas Cumhuriyet Üniversitesi Hayvan Deneyleri Yerel Etik Kurulunun 15.05.2025 tarih ve 65202830-050.04.04-34 sayılı kararıyla alınmıştır.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Konsept - TT; Tasarım - TT; Denetim - TT; Kaynaklar – TT, HI; Malzemeler – TT, HI, MOA; Veri Toplama ve/veya İşleme – TT, HI, MOA; Analiz ve/veya Yorum – TT, HI, MOA; Literatür Taraması – TT, HI, MOA; Yazma – TT, MOA; Eleştirel İnceleme – HI, MOA; Diğer – TT

Çıkar Çatışması: Yazarlar herhangi bir çıkar çatışması bildirmemektedir.

Finansal Destek: Bu çalışma için herhangi bir maddi destek alınmamıştır.

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ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.17094/vetsci. 1691935

Synergistic Antimicrobial Effects of Nisin and ε-Poly-L-Lysine on Raw Beef During Cold Storage Against Major Foodborne Pathogens

Nisin ve ε-Poli-L-Lizin'in Soğuk Depolama Sırasında Çiğ Sığır Etinde Başlıca Gıda Kaynaklı Patojenlere Karşı Sinerjik Antimikrobiyal Etkileri

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ABSTRACT

With increasing consumer demand for natural food preservatives, the use of antimicrobial substances such as nisin and epsilon-poly-L-lysine (ε-PL) in meat products has gained attention. This study aimed to evaluate the synergistic effects of nisin and ε-PL on the viability of key foodborne pathogens—*Escherichia coli* O157:H7, *Salmonella* Typhimurium (Gram-negative), and *Listeria monocytogenes* (Gram-positive)—in raw red meat. In addition, the study evaluated the impact of treatments on physicochemical characteristics and color stability. Two combinations of nisin and ε-PL were tested: Mix 1 (400 IU/g nisin + 20 µg/g ε-PL) and Mix 2 (800 IU/g nisin + 40 µg/g ε-PL), applied to raw beef samples inoculated with the pathogens. Samples were stored at 4 °C for 16 days, and microbiological (pathogen counts, total mesophilic aerobic bacteria, mold, and yeast), physicochemical (pH, water-holding capacity), and color (*L**, *a**, *b**) analyses were performed at intervals (days 0, 4, 8, 12, and 16). Mix 2 showed the most potent antimicrobial activity, decreasing *L. monocytogenes* counts to 2.15 log CFU/g by day 8. Significant reductions were also recorded for *E. coli* O157:H7 and *Salmonella* Typhimurium. Additionally, the mixtures suppressed pH increases, maintained color stability, and improved water retention. In conclusion, the free-form combinations of nisin and ε-PL effectively inhibited microbial growth, preserved meat quality, and extended shelf life, highlighting their potential as natural preservatives in the meat industry. This study provides novel evidence on the synergistic use of free-form nisin and ε-PL in raw beef, offering a valuable contribution to the development of natural preservation methods in meat products.

Keywords: Epsilon-poly-L-lysine (ε-PL), food biopreservation, meat, natural antimicrobials, nisin

ÖZ

Doğal gıda koruyucularına yönelik artan tüketici talebiyle birlikte, et ürünlerinde nisin ve epsilon-poly-L-lysine (ε-PL) gibi antimikrobiyal maddelerin kullanımı dikkat çekmiştir. Bu çalışma, nisin ve ε-PL'nin çiğ kırmızı ette gıda kaynaklı önemli patojenlerin *Escherichia coli* O157:H7, *Salmonella* Typhimurium (Gram-negatif) ve *Listeria monocytogenes* (Gram-pozitif) canlılığı üzerindeki sinerjik etkilerini değerlendirmeyi amaçlamıştır. Çalışmada ayrıca uygulamaların fizikokimyasal özellikler ve renk stabilitesi üzerindeki etkisi de değerlendirilmiştir. Nisin ve ε-PL'nin iki kombinasyonu test edilmiştir: Karışım 1 (400 IU/g nisin + 20 µg/g ε-PL) ve Karışım 2 (800 IU/g nisin + 40 µg/g ε-PL), patojenlerle inoküle edilmiş çiğ sığır eti örneklerine uygulanmıştır. Örnekler 16 gün boyunca 4 °C'de saklanmış ve aralıklarla (0, 4, 8, 12 ve 16. günler) mikrobiyolojik (patojen sayıları, toplam mezofilik aerobik bakteri, küf ve maya), fizikokimyasal (pH, su tutma kapasitesi) ve renk (*L**, *a**, *b**) analizleri yapılmıştır. Karışım 2, *L. monocytogenes* sayısını 8. günde 2,15 log CFU/g'a düşürerek en güçlü antimikrobiyal aktiviteyi göstermiştir. *E. coli* O157:H7 ve *S.* Typhimurium için de önemli azalmalar kaydedilmiştir. Ayrıca, karışımlar pH artışlarını bastırmış, renk stabilitesini korumuş ve su tutma özelliğini geliştirmiştir. Sonuç olarak, nisin ve ε-PL'nin serbest form kombinasyonları mikrobiyal büyümeyi etkili bir şekilde engellemiş, et kalitesini korumuş ve raf ömrünü uzatmıştır.

Anahtar Kelimeler: Doğal antimikrobiyaller, epsilon-poli-L-lizin (ε-PL), et, gıda biyokoruma, nisin

Received/Geliş Tarihi: 05.05.2025
Accepted/Kabul Tarihi: 08.08.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Aydemir ME, Çelen T, Altun SK, Giraz MN, Tuğbay EB, Keşküş A. Synergistic antimicrobial effects of nisin and ε-poly-l-lysine on raw beef during cold storage against major foodborne pathogens. *Vet Sci Pract*. 2025;20(2):94-103.

Atıf: Aydemir ME, Çelen T, Altun SK, Giraz MN, Tuğbay EB, Keşküş A. Nisin ve ε-poli-l-lizin'in soğuk depolama sırasında çiğ sığır etinde başlıca gıda kaynaklı patojenlere karşı sinerjik antimikrobiyal etkileri. *Vet Sci Pract*. 2025;20(2):94-103.

INTRODUCTION

Meat products are among the most widely consumed foods due to their rich nutritional profile.[1] The constant and increasing demand for meat necessitates an extensive global production and supply chain. Factors such as rapid economic growth, trade liberalization, evolving consumer lifestyles, and increasing food demand have significantly influenced the meat industry, presenting both opportunities and challenges.[1,2] However, practices adopted to meet this growing demand may compromise food safety and increase public health risks.

Due to its high-water activity, protein content, and nutrient richness, meat provides an ideal environment for microbial growth, making it susceptible to physical, chemical, and especially microbiological spoilage.[3] Microbial contamination plays a critical role in determining the safety and shelf life of meat products. During slaughter and processing, contamination can arise from various sources including poor hygiene practices, contaminated tools, animal hides, gastrointestinal contents, and transport conditions.[4] Consequently, raw meat can become easily contaminated with pathogenic bacteria, particularly during the slaughtering process, increasing the risk of serious foodborne illnesses.[1] *Salmonella Typhimurium*, *Listeria monocytogenes*, and Shiga-toxin-producing *Escherichia coli* O157:H7 are among the most implicated pathogens associated with raw red meat.[5,6] Although the food industry has invested heavily in measures to control these microorganisms, they continue to pose significant public health threats. Therefore, innovative and more effective strategies are needed to address these ongoing microbiological challenges.

Nisin, a bacteriocin produced by *Lactococcus lactis*, has received Generally Recognized as Safe (GRAS) status from the U.S. Food and Drug Administration (FDA).[7] It is widely used as a natural preservative, particularly in meat and dairy products, due to its potent antimicrobial activity, especially against Gram-positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*.[7,8]

Epsilon-poly-L-lysine (ε-PL) is a naturally occurring cationic peptide composed of L-lysine residues and is produced by fermentation using *Streptomyces albulus*. It is water-soluble, biodegradable, non-toxic, and exhibits high thermal stability. ε-PL has demonstrated broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, including *Bacillus subtilis*, *E. coli*, lactic acid bacteria, and *Staphylococcus aureus*.[9] Its mechanism of action involves binding to negatively charged bacterial cell membranes due to its cationic nature, leading to membrane disruption and cell death.[10] This unique mechanism allows ε-PL to be effective against a wide range of bacterial pathogens.

When used in combination with nisin, ε-PL has shown enhanced antimicrobial effects. Literature reports suggest that nisin + ε-PL combinations are particularly effective against Gram-positive pathogens such as *B. cereus*, *L. monocytogenes*, *S. aureus*, and *Enterococcus faecalis*, as well as some Gram-negative species including *B. subtilis* and *Lactobacillus* spp.[11] While earlier studies[12] did not report synergistic activity of this combination against *E. coli*, more recent work by[13] demonstrated promising antimicrobial effects on this pathogen. These findings underscore the need for further research to better understand the efficacy of nisin and ε-PL combinations, particularly against Gram-negative bacteria.

In this study, we aimed to evaluate the synergistic activity of nisin and ε-PL against key foodborne pathogens, specifically Gram-negative (*E. coli* O157:H7, *S. Typhimurium*) and Gram-positive (*L. monocytogenes*) bacteria, in raw red meat. Additionally, we assessed the impact of these combinations on the physicochemical and color properties of the meat.

MATERIALS AND METHODS

Ethics Committee

In accordance with Article 8(k) of the 'Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees', this study is not subject to HADYEK permission.

Preparation of mixtures of nisin and epsilon-poly-L-lysine (ε-PL)

Cationic nisin and ε-poly-L-lysine (ε-PL) were obtained commercially from Handary (Belgium). Stock solutions of nisin (0.5 mM; 1.68 mg/mL) and ε-PL (5 mM; 0.72 mg/mL) were prepared using HEPES buffer (5 mM, Sigma, UK) with low ionic strength. These solutions were subsequently filtered through 0.45 µm syringe filters to ensure sterility. To simulate the conditions of raw meat, the pH of the solutions was adjusted to 5.5, matching the typical pH level of fresh beef. Two different combinations of nisin and ε-PL were formulated: Mix 1: 400 IU/g nisin + 20 µg/g ε-PL, and Mix 2: 800 IU/g nisin + 40 µg/g ε-PL. Each mixture was stirred continuously for 4 hours using a magnetic stirrer to ensure complete homogenisation. The prepared antimicrobial solutions were subsequently applied directly to the surface of the meat samples.

Preparation of Pathogenic Bacterial İnoculum

The bacterial strains used to inoculate the meat samples included *Escherichia coli* O157:H7 (ATCC 43984), *Salmonella* Typhimurium (ATCC 14028), and *Listeria monocytogenes* (RSKK 474, 476), all obtained from the Refik Saydam National Public Health Agency (Turkey). Each strain was cultured in Tryptic Soy Broth (TSB) at 37°C for 18–24 hours. Following incubation, bacterial cells were collected by centrifuging at 4,000 rpm for 10 minutes. The obtained cell pellets were then rinsed with 0.1% peptone water to eliminate any remaining culture medium. Pellets belonging to the same species were pooled and in sterile physiological saline to prepare a uniform inoculum. Serial dilutions were prepared to obtain an inoculation level of approximately 10^5 CFU/g for each target pathogen in the meat samples.

Preparation of the Groups

Musculus longissimus dorsi from cattle slaughtered under hygienic conditions one day prior and having undergone rigor mortis was used as the meat source in this study. The meat was obtained from local butchers in Şanlıurfa Province and transported to the Food Hygiene and Technology Laboratory at Harran University, Faculty of Veterinary Medicine, under cold chain conditions. A total of 30 meat samples (15 per replicate) were used. The meat was aseptically cut into small pieces (25 ± 5 g) using a sterile scalpel. The meat samples were then experimentally inoculated with the diluted bacterial suspension. Specifically, 500 µL of the pathogen cocktail was uniformly spread across each meat sample using a sterile spreader, and samples were held for at least 10 minutes to facilitate bacterial attachment. The samples were randomly assigned into three groups: Control group (no treatment), Mix 1 (400 IU/g nisin + 20 µg/g ε-PL), Mix 2 (800 IU/g nisin + 40 µg/g ε-PL).

Each sample was placed into a 50 mL falcon tube. Then, 500 µL of the respective antimicrobial solution (prepared in HEPES buffer) was added. The tubes were shaken gently for 2 minutes to ensure uniform distribution of the treatment. After treatment, all samples were stored at 4 ± 1°C for 16 days. Microbiological, chemical, and instrumental color analyses were performed at 4-day intervals (days 0, 4, 8, 10, 12, and 16). The experiment was conducted in two independent replicates.

Microbiological Analyses

On each analysis day, meat samples were aseptically transferred from falcon tubes into sterile stomacher bags. A volume of 225 mL of 0.1% peptone water (Merck, Darmstadt, Germany) was added to each bag, and the mixture was homogenized using a stomacher for 3 minutes to obtain a 10^{-1} dilution. Serial dilutions were then prepared up to 10^{-7} using the same diluent. For microbial enumeration, the surface spread method was used for *E. coli* O157:H7, *L. monocytogenes*, and *S. Typhimurium*, while the pour plate method was employed for the total mesophilic aerobic bacteria (TMAB), molds, and yeasts. All inoculations were performed in duplicate. After incubation, microbial colonies were enumerated based on their characteristic morphology on selective media, in accordance with guidelines.[14]

Listeria monocytogenes; Enumerated on Oxford Agar (Merck, Darmstadt, Germany). After incubation at 35°C for 24–48 hours, colonies with a blackish-green to brown color, featuring black zones and sunken centers, were counted. *Salmonella Typhimurium*; Counted on Xylose Lysine Deoxycholate (XLD) Agar (Merck, Darmstadt, Germany). Colonies appearing black after incubation at 35°C for 24–48 hours were considered presumptive *S. Typhimurium*. *Escherichia coli* O157:H7; Counted using Cefixime Tellurite Sorbitol MacConkey (CT-SMAC) Agar (Merck, Darmstadt, Germany). White colonies were counted after incubation at 35°C for 24–48 hours. Total mesophilic aerobic bacteria (TMAB); Enumerated using Plate Count Agar (Merck, Darmstadt, Germany). The inoculated plates were incubated at 35°C for 24–48 hours prior to colony enumeration. Molds and yeasts; Enumerated on Dichloran Rose Bengal Chloramphenicol (DRBC) Agar (LAB 217; Lab M, Lancashire, UK). Plates were incubated at 25 ± 1°C for 5 days in accordance with ISO 21527-1.[15]

Physicochemical Analyses

pH Determination

pH measurements were conducted at 25°C using a digital pH meter (model HI 11310, Hanna Instruments, USA). Prior to each measurement, the instrument was calibrated using standard buffer solutions at pH 4.01 and 7.00. For analysis, 10 g of the meat sample was combined with 90 mL of distilled water and homogenized for 1 minute. The resulting mixture was then used for pH measurement.[16]

Water Holding Capacity

Approximately 2 g of each meat sample was placed between filter papers and placed between two glass plates (10 × 10 cm). A 10 kg weight was applied to the setup for 5 minutes. The water holding capacity was then calculated using the following equation:

$$WHC(\%)=100-[(\text{First weight}-\text{Last weight})/\text{First weight}]x100$$

Color Analysis

The color characteristics of the meat samples were determined using a digital colorimeter (model CS-10, CHNSpec, Hangzhou, China). Lightness (L^*), redness (a^*),

and yellowness (*b**) values were measured at a minimum of four different points on the outer surfaces of the samples to ensure accuracy. Prior to analysis, the colorimeter was calibrated using standard black and white reference plates.[17]

Statistical Analysis

The data obtained from the study were analysed using SPSS 24.0 (IBM Corp., Armonk, NY, USA). Microbial counts, pH, water holding capacity and colour parameters (L*, a*, b*) were subjected to statistical analysis. One-way analysis of variance (ANOVA) was employed to evaluate differences among groups and sampling days. To identify statistically significant differences, a Tukey's post hoc test was performed. All measurements were performed in duplicate in independent trials, and the data are presented as mean values accompanied by the standard error of the mean (± SE). A significance level of $P < .05$ was considered statistically significant.

RESULTS

In this study, the effects of mixtures containing different ratios of nisin and ε-PL (Mix 1 and Mix 2) on the microbiological, physicochemical, and color parameters of raw beef stored at 4 °C were evaluated. The results demonstrated that these mixtures exhibited significant antimicrobial activity.

Microbiological Results

The counts of *Listeria monocytogenes* did not show a significant change in the control group during storage ($P > .05$). However, both Mix 1 and, in particular, Mix 2 treatments exhibited a significant reduction in bacterial load. A 1-log difference between the control and treatment groups was observed on the 4th day, and an approximately 3-log difference was noted on the 8th day, with this decrease being statistically significant ($P < .05$; Table 1). Although a slight increase in the number of *L. monocytogenes* was observed in the treatment groups toward the end of storage, the difference remained statistically significant ($P < .05$), resulting in an approximately 2-log reduction relative to the control. The concentrations of nisin and ε-PL in the treatment groups did not significantly influence the inhibition of *L. monocytogenes*.

Table 1. *Listeria monocytogenes* counts (log 10 cfu/g±SE) in raw beef during storage at 4°C.a

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	5.32±0.04	$5.62±0.10^{a}$	$5.60±0.15^{a}$	$5.62±0.18^{a}$	$5.57±0.15^{a}$
Mix 1	$5.42±0.05^{A}$	$4.49±0.20A^{Bb}$	$2.65±0.24^{Cb}$	$3.66±0.22^{BCb}$	$3.72±0.19^{BCb}$
Mix 2	$5.24±0.13^{A}$	$4.30±0.23A^{Bb}$	$2.15±0.15^{Cb}$	$3.45±0.27^{Bb}$	$3.57±0.23^{Bb}$

a-b: Mean values shown with different letters in the same column are significantly different ($P < .05$). A-C: Mean values indicated by different letters in the same row are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Similarly, significant decreases in *E. coli* O157:H7 counts were observed in the Mix 1 and Mix 2 groups compared to the control group, particularly on days 8, 12, and 16 ($P < 0.05$). At the end of the 16th day, the *E. coli* O157:H7 level in the Mix 2 group was 4.82 ± 0.17 log cfu/g, which represented a difference of approximately 1 log compared to the control group (Table 2). Regarding the concentrations of nisin and ε-PL in the treatment groups, it was noted that the Mix 2 group was more effective in reducing *E. coli* O157:H7 counts, particularly on days 12 and 16.

Table 2. Escherichia coli O157:H7 counts (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	5.51±0.14	5.52±0.17	$5.53±0.27^{a}$	$5.74±0.18^{a}$	$5.84±0.15^{a}$
Mix 1	$5.67±0.10^{A}$	$5.72±0.22^{A}$	$4.83±0.12^{Bb}$	$5.50±0.10^{Aab}$	$5.19±0.12^{ABab}$
Mix 2	$5.65±0.16^{A}$	$5.47±0.26^{A}$	$4.90±0.16^{Bb}$	$5.10±0.26^{ABb}$	$4.82±0.17^{Bb}$

a-b: Mean values shown with different letters in the same column are significantly different ($P < .05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

No significant decrease in *Salmonella* count was observed in the control group; however, statistically significant reductions were recorded in the Mix 1 and Mix 2 groups throughout the entire storage period ($P < .05$). Significant decreases were particularly evident in the Mix 1 and Mix 2 groups compared to the control group, especially on days 4, 8, 12, and 16 ($P < .05$). On day 4, the Mix 2 group exhibited the lowest value at 4.55 ± 0.22 log cfu/g (Table 3). Regarding the concentrations of nisin and ε-PL in the treatment groups, it was observed that the Mix 2 group was more effective in reducing *S.* Typhimurium counts, but only on day 16.

While TMAB counts increased over time in the control group (6.62 ± 0.35 log cfu/g), the increase was more limited in the Mix 1 and Mix 2 groups, with statistically significantly lower values recorded on all days compared to the control group ($P < .05$; Table 4). The concentrations of nisin and ε-PL did not result in significant differences in TMAB counts within the treatment groups ($P > .05$). In contrast to the control group, TMAB counts in the treatment groups remained at day 0 levels even on day 16.

While mold and yeast counts increased significantly in the control group, the increase was slower in the Mix 1 and, especially, the Mix 2 groups. Notably, the Mix 2 group reached the lowest level of 1.23 ± 0.23 log cfu/g on the 8th day (Table 5).

Table 3. *Salmonella* Typhimurium counts (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	5.75±0.04	$5.40±0.18^{a}$	$5.47±0.30^{a}$	$5.57±0.18^{a}$	$5.37±0.19^{a}$
Mix 1	5.29±0.08A	$4.84±0.10^{Bb}$	$4.97±0.20^{Bb}$	$4.92±0.13^{Bb}$	$5.24±0.14^{Aa}$
Mix 2	$5.34±0.06^{A}$	$4.55±0.22^{Bb}$	$4.72±0.15^{Bb}$	$4.88±0.17^{Bb}$	$4.90±0.12^{Bab}$

a-b: Mean values shown with different letters in the same column are significantly different ($P <.05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P <.05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Table 4. Total mesophilic aerobic bacteria (TMAB) count (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	$5.55±0.11^{B}$	$5.91±0.06^{ABa}$	$6.19±0.15^{Aa}$	$6.48±0.18^{Aa}$	$6.62±0.35^{Aa}$
Mix 1	$5.54±0.10^{A}$	$4.97±0.16^{Bb}$	$5.58±0.14^{Ab}$	$5.80±0.11^{Ab}$	$5.42±0.27^{Ab}$
Mix 2	$5.40±0.15^{A}$	$4.93±0.23^{Bb}$	$5.42±0.15^{Ab}$	$5.92±0.25^{Ab}$	$5.70±0.25^{Ab}$

a-b: Mean values shown with different letters in the same column are significantly different ($P < .05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Table 5. Mold and yeast count (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	$1.27±0.14^{B}$	$2.38±0.21^{Aa}$	$2.60±0.27^{Aa}$	$2.45±0.15^{Aa}$	$2.40±0.25^{Aa}$
Mix 1	$1.71±0.13^{A}$	$1.23±0.23^{Bb}$	$1.76±0.33^{Ab}$	$1.87±0.17^{Ab}$	$1.97±0.23^{Aa}$
Mix 2	$1.66±0.26^{A}$	$1.20±0.20^{Bb}$	$1.23±0.23^{Bc}$	$1.77±0.20^{Ab}$	$2.03±0.28^{Aa}$

a-b: Mean values shown with different letters in the same column are significantly different ($P <.05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P <.05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Physicochemical Results

In the control group, the pH value increased over time, reaching 6.40 ± 0.19 by the end of the 16th day ($P <.05$). In contrast, pH changes were less pronounced in the Mix 1 and Mix 2 groups, and a significant difference was observed between the control and treatment groups on day 16 ($P <.05$, Figure 1). This suggests that the applied mixtures effectively suppressed microbial activity and prevented pH increases. No significant differences in water holding capacity were observed between the groups throughout the storage period ($P >.05$), nor were there any significant differences between the treatment groups ($P >.05$; Figure 1).

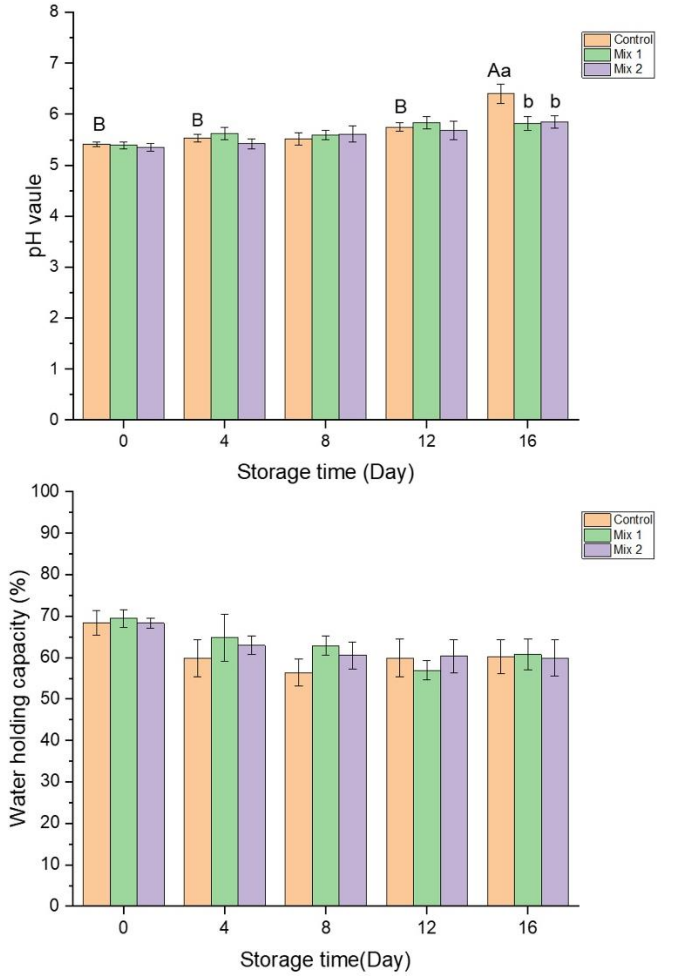

Figure 1. pH value and water holding capacity of raw beef during storage at 4 °C (Mean ±SE). a-b: Mean values indicated by different letters between groups, A-B: Mean values indicated by different letters between sampling days are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

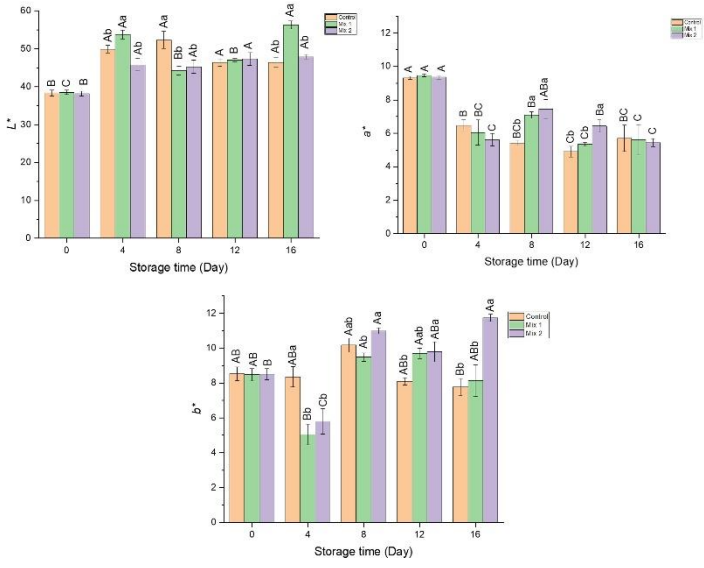

Figure 2. Color values of raw beef during storage at 4°C (Mean ±SE). a-b: Mean values indicated by different letters between groups, A-C: Mean values indicated by different letters between sampling days are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Color Parameters

In terms of the *L** value, a significant increase (53.79 ± 1.16) was observed, particularly on day 4 in the Mix 1 group, compared to the control group (P <.05). The *a** value decreased over time, but it remained higher in the Mix 2 group on day 8 (7.44 ± 0.58) compared to the control group (P <.05; Figure 2). The *b** value was significantly higher in the Mix 2 group on days 8 and 16 (e.g., day 16: 11.73 ± 0.20, P <.05; Figure 2). These results suggest that the combination of nisin and ε-PL may positively influence the color stability of meat.

DISCUSSION

In the present study, the application of combined nisin and ε-PL treatments, particularly Mix 2 (800 IU/g nisin + 40 µg/g ε-PL), significantly reduced the counts of *Listeria monocytogenes* in raw beef, reaching 2.15 log cfu/g by day 8. This result aligns with the findings of Zimet et al.[18], who demonstrated that free-form nisin effectively reduced *L. monocytogenes* levels in lean beef. The slight increase in *L. monocytogenes* numbers in the treatment groups towards the end of storage can be attributed to the fact that free-form nisin and ε-PL are effective for a limited period. As noted in previous studies, free nisin is influenced by the food matrix, and significant decreases in its activity and stability are observed when applied in its free form to food.[19]

The data obtained for *E. coli* O157:H7 once again demonstrated the limited effect of nisin against Gram-negative bacteria, while ε-PL partially compensated for this limitation. In the Mix 2 group, the counts decreased to 4.82 log cfu/g by day 16, whereas the decrease in Mix 1 was more limited. This difference is likely due to the lower concentrations of nisin and ε-PL in Mix 1, which resulted in a more limited duration of action and reduced stability in the meat matrix.[19] Furthermore, although ε-PL is known to be an effective antimicrobial agent against Gram-negative bacteria, no significant reduction in *E. coli* O157:H7 was observed, which may be due to the insufficient concentration of ε-PL applied.

The effects on *S.* Typhimurium were evaluated similarly. Although a significant decrease (4.55 log cfu/g) was achieved in the early period (day 4) in the Mix 2 application, the continuity of this effect was limited in the following days. This reduction in effectiveness may be due to interactions between free-form antimicrobials and food matrix components, which reduce their stability and bioavailability. Similar to the results observed for *E. coli* O157:H7, both free nisin and free ε-PL demonstrated antimicrobial activity against *S. Typhimurium*, another

Gram-negative pathogen in meat samples, with a reduction in Salmonella count of approximately 1 log in the first 8 days of preservation. However, this effect was not observed in the remaining days, likely due to the decrease in the stability of these antimicrobials over time.

In the control group, pathogenic bacteria counts appeared relatively stable throughout the storage period. This observation can be explained by several intrinsic factors associated with raw beef. Cold storage at 4±1°C is known to suppress bacterial growth and delay the proliferation of pathogens by slowing down metabolic activity and prolonging the microbial lag phase.[20] In addition, the presence of natural microflora in raw meat, particularly lactic acid bacteria (LAB), may have exerted a slight antagonistic effect on inoculated pathogens. These naturally occurring microorganisms may compete for essential nutrients and surface attachment sites or produce inhibitory compounds such as organic acids and bacteriocins, thereby limiting the growth of exogenous pathogens.[21] Although background flora was not quantitatively analysed in this study, it is possible that microbial interference contributed to the observed stabilization of pathogen populations in untreated control samples, especially in the early stages of storage.

The literature provides various reports on the effectiveness of nisin and ε-PL against important foodborne pathogens. These antimicrobials are shown to be effective at different levels.[22-27] The variations in effectiveness between studies can be attributed to factors such as bacterial strain, application time, method, antimicrobial concentration, and food type. Most notably, the antibacterial activity of these antimicrobials may be influenced by the composition of the food matrix. In fact, a previous study[22] reported that ε-PL exhibited a more pronounced bacteriostatic effect in rice and vegetable extracts than in milk, beef, or sausage extracts, which are rich in protein content. Furthermore, it has been reported that the antibacterial effect of ε-PL can be altered by its interaction with food components, forming a compound with a different charge, which reduces its ability to interact with anionic microbial surfaces and diminishes its antibacterial activity against *E. coli*.[28] Therefore, while nisin and ε-PL in free form are effective against foodborne pathogens, their efficacy is limited. To enhance their effectiveness in food applications, they should be supported by encapsulation technologies that enable controlled release systems.

The acceptable upper limit for total viable counts in meat and meat products is generally considered to be around 7.0 log10.[20] While TMAB counts in the control group approached this limit on day 12 (6.62 log cfu/g), they remained significantly lower in the Mix 1 and Mix 2 groups. These findings align with similar studies; in particular, it has been reported that ε-PL exhibited a strong antimicrobial effect in meat and meat products, significantly reducing TMAB counts.[26,29] In contrast, a reduction of around 0.5 log CFU/g in TMAB counts was observed in sausages packaged with nisin activity.[26] In Mix 1 and Mix 2 groups, TMAB counts decreased on day 4 and then increased again (Table 4). These fluctuations were also observed in mesophilic pathogenic bacteria (*S.* Typhimurium and *E. coli* O157:H) (Table 2, Table 3). However, such a biphasic growth pattern is not unusual and may be explained by microbial stress adaptation dynamics. Immediately after cold storage and exposure to antimicrobials, bacterial cells can enter a state of cold shock or non-lethal injury, which temporarily reduces their culturability. As storage progresses and the bacteria adapt to the cold environment and antimicrobials, the injured cells can repair their membranes and enzyme systems, re-enter active growth and thus rise again at later stages.

Meat and meat product surfaces are particularly vulnerable to contamination by molds and yeasts, leading to deterioration in both quality and sensory characteristics. In the present study, it was observed that the antimicrobial treatments reduced the number of yeasts and molds in the treatment groups until the 8th day of preservation. This result supports the antifungal potential of the antimicrobial mixtures. The findings are consistent with studies showing that polyethylene films combined with nisin (400-800 IU/g) exhibit significant antimicrobial activity against yeasts and molds in cutlets during storage[30], and that nisin and ε-PL have notable antimold activity in packaged sausages.[26]

When the pH values were analysed, significant increases were observed in the control group by the 16th day (6.40), while the pH values remained stable around 5.8 in the Mix 1 and Mix 2 groups. The higher pH in the control sample can be attributed to bacterial growth, particularly the production of lactic acid by lactic acid bacteria, as well as the inhibition of protein degradation and the formation of nitrogenous compounds by the antimicrobial agents, which suppress microbial activity.[31,32] In fact, TMAB numbers increased significantly in the control group compared to the other groups (Table 5). The increase in bacterial population leads to a rise in bacterial enzyme activity in the meat tissue, where these enzymes break down meat proteins and produce nitrogenous compounds, ultimately causing an increase in pH.[32] Although there was no statistically significant difference between the groups in terms of water holding capacity (WHC), it is noteworthy that higher values were recorded in the Mix 1 group on the 4th and 8th days. This suggests that the applied antimicrobial mixture may

indirectly help preserve the water holding capacity of meat by reducing protein denaturation.[33] The more stable pH values also support this outcome, as water loss tends to increase when proteins approach their isoelectric point.[34]

When the color parameters were examined, the values for *L**, *a** and *b** varied during storage. In terms of *L** values, it was observed that the Mix 1 application contributed to the formation of lighter colored meat, especially in the initial period. The *a** (redness) value was highest in the Mix 2 group on the 8th day (7.44); this could be linked to the suppression of lipid oxidation and the delayed formation of metmyoglobin. A decrease in *a** values is often considered an indicator of myoglobin oxidation and metmyoglobin formation, which causes the meat to turn brown.[35] On the 8th and 12th days, the *a** value in the treatment groups was higher than in the control group (Figure 2), demonstrating the antimicrobials' ability to delay the formation of metmyoglobin and thus preserve the color quality of the meat for a longer period.[31] The *b** value also increased significantly in the Mix 2 group, with the treatment groups showing higher *b** values than the control, particularly towards the end of the preservation period (Figure 2). This may be related to the antimicrobial effects of the treatment. A decrease in the *b** value is typically associated with reduced oxymioglobin content and increased metmyoglobin formation, with the decline in oxymioglobin attributed to oxygen consumption by microorganisms.[35] Additionally, this increase in *b** value could be due to the color changing properties of the antimicrobials themselves. Indeed,[26] emphasized that the high *b** value in sausages was due to the yellow color of the antimicrobial agents used.

In conclusion, this study evaluated the effects of free-form combinations of the natural antimicrobial agents nisin and ε-PL on major foodborne pathogens, including *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium. The findings demonstrated that the combination of nisin and ε-PL in free form exhibited significant antimicrobial activity when applied in specific ratios. Notably, the treatments effectively suppressed *L. monocytogenes*, with Mix 2 (higher concentration) showing considerable activity against *E. coli* O157:H7 and *S.* Typhimurium by the end of the storage period. In addition, the antimicrobial mixtures helped to limit pH increases, preserve water holding capacity, and maintain stable color characteristics in the meat. These results suggest that nisin and ε-PL, when combined in appropriate ratios, can delay microbial spoilage and help maintain the quality of raw meat products. However, to prolong their effectiveness, these compounds should be coupled with encapsulation technologies. Future research on their application across various meat types, alongside packaging solutions, consumer acceptance, and sensory evaluations, will provide further insights into their potential for enhancing food safety and quality.

Ethics Committee Approval: This study did not involve live animals, and an ethics committee decision was not required. Furthermore, animal experiments are not subject to HADYEK approval in accordance with Article 8(k) of the Regulation on the Working Principles and Procedures of Animal Experimentation Ethics Committees. Reference: https://www.resmigazete.gov.tr/eskiler/2014/02/20140215-6.htm

Informed Consent: There is no situation that requires Participant Consent in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – MEA,TÇ; Design – MEA,TÇ; Supervision – MEA,TÇ; Resources – MEA,TÇ; Materials – MEA,TÇ; Data Collection and/or Processing – MEA,TÇ,MNG,EBT,AK; Analysis and/or Interpretation – MEA,TÇ,MNG; Literature Search – MEA,EBT; Writing Manuscript – MEA,SKA; Critical Review – MEA,TÇ,SKA

Declaration of Interests: The authors declare that there is no conflict of interest.

Funding: The authors declared that they received no financial support for this study.

Etik Komite Onayı: Bu çalışmada canlı hayvanlar üzerinde bir çalışma yapılmamış olup, etik kurul kararına gerek duyulmamaktadır. Ayrıca Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik" Madde 8 (k) uyarınca HADYEK iznine tabi değildir. Kaynak: https://www.resmigazete.gov.tr/eskiler/2014/02/20140215-6.htm

Aydınlatılmış Onam: Çalışmada Katılımcı Onayı gerektiren bir durum yoktur.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Konsept – MEA,TÇ; Tasarım – MEA,TÇ; Denetim – MEA,TÇ; Kaynaklar – MEA,TÇ; Malzemeler – MEA,TÇ; Veri Toplama ve/veya İşleme – MEA,TÇ,MNG,EBT,AK; Analiz ve/veya Yorum – MEA,TÇ,MNG; Literatür Taraması – MEA,EBT; Yazma – MEA,SKA; Eleştirel İnceleme – MEA,TÇ,SKA

Çıkar Çatışması: Yazarlar herhangi bir çıkar çatışması olmadığını beyan etmişlerdir.

Finansal Destek: Yazarlar bu çalışma için herhangi bir finansal destek almadıklarını beyan etmişlerdir.

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ATATURK UNIVERSITY PUBLICATIONS

Case Report *Olgu Sunumu* DOI: 10.17094/vetsci.1555729

A Case of Generalized Demodicosis and Pyoderma Caused by *Enterobacter cloacae* in a Pug Dog

Pug Irkı Bir Köpekte *Enterobacter cloacae* Kaynaklı Piyoderma ve Generalize Demodikozis Olgusu

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ABSTRACT

This case aimed to evaluate the role of *Enterobacter cloacae (E. cloacae*), a rare gram-negative bacterium, in the etiology of pyoderma and generalized demodicosis in a 7-month-old Pug. *Demodex canis (D. canis)* and *E. cloacae* were isolated from deep skin scraping and biopsy samples. Miticidal treatment included afoxolaner and milbemycin oxime (2.5–5 mg/kg and 0.5–1 mg/kg, respectively). Antibacterial therapy consisted of cefazolin (20 mg/kg, IV, BID) and benzylpenicillin + dihydrostreptomycin (0.5 mL/5 kg, IM, SID) until culture and sensitivity results were obtained. The dog was returned to its shelter, where treatment was continued by the veterinarian and owner. Unfortunately, it died on the third day post-treatment. This case highlights that rare pathogens such as *E. cloacae* can contribute to severe, refractory demodicosis with secondary pyoderma. Pending antibiogram results, using less commonly employed antibiotics may help address resistance. Regular bacterial cultures and antibiograms are essential for identifying both common and rare pathogens and monitoring their resistance profiles, thus improving treatment success in complex dermatological cases.

Keywords: *Enterobacter cloacae*, generalized demodicosis, pug, pyoderma

ÖZ

Bu olgu sunumunda, 7 aylık Pug ırkı bir köpekte piyodermanın etiyolojisinde nadir olarak görülen Gram negatif bir bakteri olan *Enterobacter cloacae* (*E. cloacae*) ve generalize demodikozis değerlendirildi. Parazitolojik ve bakteriyolojik incelemeler sonucunda derin deri kazıntısı ve biyopsi örneklerinden *Demodex canis (D. canis)* ve gram negatif bir bakteri *(E. cloacae)* izoleedilerek identifiye edildi. Mitisidal tedavi amacıyla afoksolaner + milbemisin oksim (2.5-5 mg/kg afoksolaner + 0.5-1 mg/kg milbemisin oksim, Nexgard Spectra® 3.5-7.5 kg Köpekler için Çiğnenebilir Tablet, Boehringer Ingelheim) kombinasyonu kullanıldı. Antibakteriyel tedavi için bakteriyel kültür ve antibiyogram sonuçları çıkana kadar sefazolin (20 mg/kg dozda, IV, günde iki kez) ve benzilpenisilin + dihidrostreptomisin (0,5 mL/5 kg dozunda, IM, günde bir kez) kombinasyonu kullanıldı. Hayvan sahibi tarafından köpek kendi barınağına götürüldü. Tedavisi buradaki veteriner hekim ve hayvan sahibi tarafından takip edildi. Tedavi sonrası 3. günde köpeğin ex olduğu öğrenildi. Sonuç olarak, klinisyenler tedaviye yanıt vermeyen, inatçı, piyoderma ile komplike generalize demodikozis vakalarının etiyolojisinde *E. cloacae* gibi nadir olarak görülebilen bakterilerin var olabileceğini göz önünde bulundurmalıdır. Antibiyogram sonuçları çıkana kadar rezistans sorununu ekarte edebilmek için, klinik rutinde nadir olarak kullanılan antibiyotiklerin tercih edilmesi, zaman zaman bakteri kültürü ve antibiyogram yapılarak yaygın ve nadiren izole edilmiş bakterilerin tanımlanması ve bu bakterilerin direnç profillerinin ortaya çıkarılmasının önemli olduğunu göstermektedir.

Anahtar Kelimeler: *Enterobacter cloacae*, generalize demodikozis, pug, piyoderma

Received/Geliş Tarihi: 25.09.2024
Accepted/Kabul Tarihi: 04.07.2025
Publication Date/Yayın Tarihi: 27.08.2025

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Cite this article: Ekinci G, Bekdik İK, Tüfekçi E, et al. A case of generalized demodicosis and pyoderma caused by *Enterobacter cloacae* in a pug dog. *Vet Sci Pract*. 2025;20(2):104-109.

Atıf: Ekinci G, Bekdik İK, Tüfekçi E, et al. Pug Irkı Bir Köpekte *Enterobacter cloacae* kaynaklı pyoderma ve generalize demodikozis olgusu. *Vet Sci Pract*. 2025;20(2):104-109.

INTRODUCTION

Dogs are frequently affected by the parasitic skin condition demodicosis, which is characterized by an excessive number of *Demodex* mites on the skin's surface and in hair follicles.[1] Clinical signs of demodicosis include papules, pustules and draining tracts with alopecia, comedones, follicular scaling, hyperpigmentation and secondary bacterial infection.[2] One of the most common complication of generalized demodicosis is a secondary bacterial infection of skin (superficial or deep pyoderma, folliculitis, furunculosis).[1] Bacterial septicemia is seen especially in severe cases involving frunculosis. *Staphylococcus intermedius* is the most common infecting organism, but secondary infection with *Pseudomonas aeruginosa* or *Proteus mirabilis* may also occur.[3]

Enterobacter cloacae (E. cloacae) are gram-negative bacteria that belong to the family Enterobacteriaceae.[4] They can be both aerobic and anaerobic. *E. cloacae* is a component of the normal intestinal flora of many mammals.[5] However, *E. cloacae* can cause wound, respiratory and urinary tract infections.[6]

This case was discussed to bring to the attention of our colleagues about a case that we encounter from time to time in our clinics and sometimes have difficulty in treating. In this case report, generalized demodicosis complicated with pyoderma caused by *E. cloacae*, a gram-negative bacterium that is rarely seen in the etiology of pyoderma in a Pug breed dog, was evaluated.

CASE PRESENTATIONS

The material of the case was an 8-month-old, male, 4.8 kg body weight, Pug breed dog brought to Erciyes University, Faculty of Veterinary Medicine, Veterinary Teaching Hospital, Small Animal Clinic with complaints of loss of appetite, severe itching, hair loss, thickening of the skin and purulent discharge from the skin. It was learned that the dog was owned and kept in their garden of the house. It was determined that the dog was fed with leftover food as a diet and occasionally given dry food. It is known that there are no vaccinations and antiparasitic drugs are not done regularly. It was reported that the disease was noticed 17 days ago and that it first started as a small wound in the head area and then spread to other parts of the body. It was learned that praziquantel + ivermectin, fipronil and *Tarantula cubensis* D6 were used in the treatment. Clinical examination of this dog, which was brought to our hospital after the disease did not improve, revealed alopecia, itching, hyperpigmentation in the chest, armpit, abdomen and inguinal areas, pustule formation and crusting, hyperkeratosis, foul-smelling and purulent discharge. Additionally, pododemodicosis was present in this case (Figure 1 A-B). When the physical examination findings were examined, it was determined that the dog was depressed and cachectic, its body temperature was 36.0°C, respiratory frequency was 20/min and pulse frequency was 144 beats/min. Mild paleness was detected in the mucosa. Capillary refill time (CRT) was measured at 4 seconds, supporting the clinical estimation of approximately 8% dehydration, consistent with moderate to severe dehydration. No endoparasite eggs were seen in the native examination of the stool.

Complete blood count revealed leukocytosis (29.4 $\times 10^9$/L), lymphocytosis (6.3 $\times 10^9$/L), neutrophilia (21.8 $\times 10^9$/L) and normocytic normochromic anemia [RBC (3.22 $\times 10^{12}$/L), Hgb (6.9 g/dL), Hct (19.9%)]. Interestingly, both the absolute eosinophil count and the eosinophil percentage were not elevated and were reported as zero (Table 1).[7]

Table 1. Complete blood count results

Variables		Ref. Ranges[7]
WBC (10^9/L)	29.4 H	6.00-17.00
Lymph (10^9/L)	6.3 H	0.90-5.00
Mono (10^9/L)	1.3	0.30-1.50
Neut (10^9/L)	21.8 H	3.50-12.00
Eos (10^9/L)	0.0	0.10-1.50
Lymp (%)	21.5	9.00-47.00
Mono (%)	4.2	2.00-12.00
Neut (%)	74.3	42.00-84.00
Eos (%)	0.0	1.00-18.00
RBC (10^{12}/L)	3.22 L	5.50-8.50
Hgb (g/dL)	6.9 L	12.00-18.00
Hct (%)	19.9 L	37.00-55.00
MCV (fL)	61.8	60.00-72.00
MCH (pg)	21.5	19.50-25.50
MCHC (g/dL)	34.8	32.00-38.50
RDWa (fl)	46.7	35.00-65.00
RDW (%)	16.3	12.00-17.50
PLT (10^9/L)	187	200.00-500.00
MPV (fL)	6.8	5.50-10.50

RBC; Red Blood Cell, Hct; hematocrit, Hgb; hemoglobin concentration, Lymph; lymphocyte, Neut; Neutrophil, Mono; monocyte, Eos; Eosinophil, MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin volume, MCHC; mean corpuscular hemoglobin concentration, WBC; White Blood Cell, PLT; platelet, RDW; Red cell distribution, RDWa; absolute value of the width of the distribution of red blood cells, MPV; mean platelet volume. H: High, L: Low. [7]Canine and feline hematology reference values.

Figure 1. A Pug dog with generalized demodicosis complicated with pyoderma with **(A)** purulent discharge on the face and **(B)** hair loss, crusting, and hyperkeratosis in other parts of the body

Biochemical analysis showed an increase in BUN (66.82 mg/dL), ALP (208 U/L), GGT (12 U/L) enzyme activity and phosphorus (9.2 mg/dL) levels. AST (5.9 U/L) enzyme activity, albumin (1.89 g/dL), and sodium (138.8 mmol/L) levels were found to be lower than normal values (Table 2).[8]

Table 2. Biochemical analysis results

Variables		Ref. Ranges[8]
BUN (mg/dL)	66.82 H	19 - 34
Creatinine (mg/dL)	0.77	0.9 - 2.2
Total Protein (g/dL)	6.42	6.0 - 7.9
Albumin (g/dL)	1.89 L	2.8 - 3.9
Glukoz (mg/dL)	84.55	60 - 120
ALP (U/L)	208 H	0 - 45
ALT (U/L)	13.6	0 - 45
AST (U/L)	5.9 L	7 - 38
GGT (U/L)	12 H	0 - 10
CK (U/L)	314	52–368
Total Bilirubin (mg/dL)	0.43	0-0.2
LDH (U/L)	124.8	0–236
Na (mmol/L)	138.8 L	146 - 156
K (mmol/L)	4.18	3.7 - 6.1
Ca (mg/dL)	10.43	8.7 - 11.7
P (mg/dL)	9.2 H	3.0 - 6.1
Mg (mg/dL)	2.36	1.7 - 2.6

ALT; alanine aminotransferase, ALP; alkaline phosphatase, AST; aspartate aminotransferase; CK; creatine kinase, GGT; gamma glutamyltransferase, LDH; lactate dehydrogenase, CRP; C-reactive protein, BUN; blood urea nitrogen, CK; creatine kinase, Na; sodium, K; potassium, Cl; chlorine, Ca; calcium, P; phosphorus, Mg; magnesium.

For parasitological examination, deep skin scraping (until capillary bleeding occurs) samples were taken from 4 different parts of the body (head, right front extremity, right hind extremity, ventral abdominal wall). These skin scraping samples were treated with 10% KOH and waited for 10 minutes. Then, *D. canis* agents were imaged using 10× and 40× objectives under the light microscope with camera attachment (Olympus BX-50, Japan). To calculate the number of adults, larvae, nymphs, and eggs in the scraping samples taken from each region, 10 microscope fields were examined at 10X magnification in the preparation obtained from each scraping, and the total number of agents obtained was divided by 10, and the average was calculated.[9] Many *D. canis* agents were seen in these samples (Figure 2). Average mite numbers are as follows; adult (45), larva (15), nymph (102), eggs (48). Since the dog died on the 3rd day of treatment, mite numbers could not be determined after treatment (day 28, day 56) (Table 3).

Figure 2. Demodex canis mites

Adhesive tape technique was used for cytological examination and Giemsa staining was performed. Mallezzia organisms were seen on the slide, although not very densely.

Table 3. *D. canis* mite numbers

	Ventral abdominal	Right Forelimb	Right hindlimb	Head region	Mean
Adult	84	44	28	22	45
Larva	12	22	14	12	15
Nymph	48	20	24	10	102
Eggs	26	2	10	10	48

A sample was taken from the abscess in the head area for bacteriological culture and antibiogram. Before taking the sample, the hair in the area was shaved and the area was disinfected with 70% ethyl alcohol and povidone-iodine solution. Samples were placed in separate sterile culture

tubes as appropriate for aerobic or anaerobic culture. The samples were quickly sent to the bacteriology laboratory under appropriate transportation conditions. The transplanted samples were incubated in appropriate media under both aerobic and anaerobic conditions at 37°C for 48-72 hours. The sample sent to the laboratory was inoculated on 5% Sheep Blood Agar, Mannitol Salt Agar and Mac Conkey Agar. After the incubation period, colony morphologies were examined. Suspicious isolates, whose gram characteristics were determined, were uploaded to the automated microbiology testing system (BD Phoenix 100, Biosciences, USA) for bacterial identification and antibiogram (MIC). In addition, an antibiogram was performed using the disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) standards for antibiotics more commonly used in veterinary medicine. The results were evaluated and reported by experienced and expert personnel. As a result of bacteriological cultivation, only *E. cloacae* was isolated and identified. As a result of the antibiogram, it was determined that the agent was resistant to 17 of 33 types of antibiotics, sensitive to 13, moderately sensitive to 2 and slightly sensitive to 1.

Antibiogram results as follows. Sensitive; amikacin, gentamicin, ertapenem, imipenem, meropenem, ceftazidime, ceftriaxone, cefepime, ceftolozane+tazobactam, piperacillin+tazobactam, ciprofloxacin, levofloxacin, trimethoprim+sulfamethoxazole. Less sensitive; streptemycin. Moderate sensitive; marbofloxacin, enrofloxacin, Resistant; cefazolin, ampicillin, amoxicillin+clavulanate, ampicillin+sulbactam, colistin, bacitracin, neomycin, kanamycin, cephalexin, moxifloxacin, peniciline, tigecycline, amoxicillin, lincomycin+spectinomycin, oxytetracycline, doxycycline, cefuroxime.

For miticidal treatment, afoxolaner and milbemycin oxime (orally every 30 days, at the recommended dosage of 2.5-5 mg/kg of afoxolaner and 0.5-1.0 mg/kg of milbemycin oxime was used.[8] For antibacterial treatment, cefazolin (at a dose of 20 mg/kg IV twice daily for 7 days) and benzylpenicillin+dihydrostreptomycin (at a dose of 0.5 mL /5 kg B.W. /day IM for 7 days) until bacterial culture and antibiogram results are available. They were advised to bathe twice a week with a shampoo containing benzoyl peroxide. Omega-3, Omega-6, Vitamin B_1, Vitamin B_2, Vitamin B_6, Vitamin B_{12}, Biotin and Zinc (oral, once a day, 1 capsule) were prescribed as supportive treatment. The dog was taken to its own shelter by the owner. Additionally, fluid electrolyte therapy was administered. The total amount of fluid (672 mL) to be administered was calculated by taking into account the dehydration deficit (4.8 kg × 0.08 × 1000 = 384 mL) and the daily maintenance fluid requirement (4.8 kg × 60 = 288 mL/day). Since no vomiting or diarrhea was observed, possible ongoing losses were taken as 0 mL. Intravenous fluids were administered at a rate of 60 mL/kg/hour. In the first six hours, 384 mL (to make up for the lack of water) was given, and the last 288 mL was slowly given over in the next 16 hours. The treatment included: isotonic saline (0.9% NaCl), 5% Dextrose solution and Ringer's lactate in combination. Fluid and electrolyte therapy was planned to be continued for at least 3 days to ensure complete rehydration and stabilisation. His treatment was followed jointly by the veterinarian there and the animal owner. It was learned that the dog died on the 3rd day after the treatment.

DISCUSSION

Juvenile demodicosis is more common in pure bred dogs of particular breeds.[2] Although the disease is relatively easy to diagnose, it is often difficult to treat. In fact, in some cases, especially in the generalized form complicated by pyoderma, the prognosis can be poor. The generalized form can be one of the most severe canine skin diseases, and can be life threatening if not treated adequately and promptly.[10,11]

In this case, oral miticidal therapy was started despite the patient being systemically unstable. This decision was made considering the severity of the parasitic load and the need to rapidly control *D. canis* infestation. The treatment protocol included afoxolaner and milbemycin oxime. Both compounds are broadly recognized miticidal agents demonstrated to be safe and efficacious, even in clinically compromised animals.[2,12] Although caution is generally recommended when using oral medications in systemically unstable patients, in conditions such as generalized demodicosis, early and effective miticidal intervention is critical to preventing secondary infections, systemic inflammation, and clinical deterioration. Therefore, oral miticide use has been considered as part of a supportive care plan, and the patient is carefully monitored throughout treatment.

A study found that owners of American Staffordshire terriers, Staffordshire bull terriers, Chinese shar-pei, and French bulldogs had a more than four-fold increased chance of acquiring generalized demodicosis.[13] The English bulldog, Pit bull, and Sealyham terrier are breeds that are prone to juvenile onset demodicosis, according to a more extensive study conducted in the United States.[14] However, no information has been found regarding the susceptibility of Pug breed dogs to generalized demodicosis. Only one

report reported that Pug puppies were safely and effectively treated with doramectin at a dose of 0.6 mg/kg/week, with clinical lesions resolving within four weeks and being mite negative by eight weeks.[15] A 12-year-old female pug mix dog was reported to have Demodicosis with *Malassezia* sp.[16] However, according to our clinical experience, cases of generalized demodicosis accompanied by pododemodicosis are common in these breeds.[1,15,16]

In this case report, generalized demodicosis (juvenile-onset) complicated with pyoderma was described in a 7-month-old Pug puppy. Additionally, pododemodicosis was also present in this case. In humans, two cases of gram-negative bacteria have been reported, one of which was a 17-year-old adolescent male, consistent with pyoderma faciale.[17] *E. cloacae* was found in one patient and *Klebsiella oxytoca* was found in the other.[17] Shumaker et al.[18] reported that deep bacterial infection was present in 29 (94%) of 31 cases of acral lick dermatitis (ALD) in dogs. *E. cloacae* was identified in 2 of these 29 dogs diagnosed with acral lick dermatitis and deep bacterial infection in the skin. It has been reported that these isolated Enterobacter species are highly resistant microorganisms.[18] In the current case report, pyoderma caused by *E. cloacae* accompanying generalized demodicosis was determined. It was observed that the infection was very severe and even two of the signs of sepsis (leukocytosis, hypothermia) were present. On the other hand, in another study, *Escherichia coli* and other Enterobacteria such as *Klebsiella pneumoniae, Proteus mirabilis, Raoultella ornithinolytica, E. cloacae, Serratia marcescens* and *Citrobacter youngae* were reported as the predominant Gram-negative bacterial species isolated from skin infections in dogs.[19] Additionally, a multidrug resistance profile was detected in 64% of Enterobacter strains.[19] In the current case report, antibiotics cefazolin and penicillin + streptomycin combination were initially used. Supportive drugs containing Omega-3, Omega-6, Vitamin B_1, Vitamin B_2, Vitamin B_6, Vitamin B_{12}, biotin and zinc are used to correct dry and dull hair, hair loss and dermatological problems that occur on the skin.[1,11] In this case, supportive treatment was applied in addition to the main treatment. As a result of the antibiogram, it was determined that the identified *E. cloacae* was resistant to cefazolin and penicillin and less sensitive to streptomycin. Additionally, as a result of the antibiogram, it was determined that *E. cloacae* was also resistant to 17 of 33 antibiotics. When the antibiogram result came out 3 days later, it was learned that the dog was dead. Therefore, failure to control the infection due to antibiotic resistance may have caused the case to die.

Nowadays, isoxosaline group antiparasitic drugs such as afoxolaner, fluralaner and sarolaner are used in the treatment of generalized demodicosis.[1] Impressive results from the combination of afoxolaner + milbemycin oxime have been reported by many researchers.[20,21] In this case, afoxolaner at a dose of 2.5 mg/kg and milbemycin oxime at a dose of 0.5 mg/kg were used orally. However, the effectiveness of the drug could not be determined because the patient died on the 3rd day of treatment.

As a result, improved antibiotic stewardship seems necessary to stop the global spread of antibiotic-resistant Enterobacteriaceae strains. Thanks to the findings obtained from this case, it becomes clear that clinicians should pay attention to antibiotic selection in cases of generalized demodicosis complicated with pyoderma that is refractory to treatment and persistent. Additionally, until the antibiogram results are available, it is important to choose antibiotics that are rarely used in clinical routine (low risk of resistance), to identify common and less commonly isolated bacteria by performing bacterial culture and antibiogram from time to time, and to reveal the resistance profiles of these bacteria.

Ethics Committee Approval: There are no ethical issues with this study.

Informed Consent: Verbal consent was obtained from the owner of the dog whose opinions were consulted in the study for the use of this information in the article.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – G.E., İ.K.B.; Design – E.T., G.E.; Supervision – İ.K.B.; Resources – S.U., Ş.Ö., C.B.; Materials – N.B.K., M.A., Z.K.; Data Collection and/or Processing – Ş.Ö., C.B.; Analysis and/or Interpretation – G.E., Ö.D., A.Y.; Literature Search – G.E.; Writing Manuscript– G.E., C.B.; Critical Review – İ.K., Ö.D., A.Y.

Declaration of Interests: The authors declare that they have no conflict of interest.

Funding: The author(s) received no financial support for the research, authorship, and/or publication of this article

Animal Welfare: The authors certify that, in order to protect animals used in research, they have followed the ARRIVE Guidelines.

Etik Komite Onayı: Bu çalışmada herhangi bir etik sorun bulunmamaktadır.

Katılımcı Onamı: Çalışmada fikirlerine başvurulan köpeğin sahibinden bu bilgilerin makalede kullanılması için sözlü onam alınmıştır.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Fikir - G.E, İ.K.B.; Tasarım - ET., G.E.; Denetleme - İ.K.B.; Kaynaklar - S.U., Ş.Ö., C.B.; Malzemeler - N.B.K, M.A., ZK.; Veri Toplanması ve/veya İşleme - Ş.Ö., C.B.; Analiz ve/veya Yorum - G.E., Ö.D., A.Y.; Literatür Taraması - G.E.; Yazıyı Yazan - G.E., C.B.; Eleştirel İnceleme - İ.K., Ö.D, A.Y.

Çıkar Çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

Finansal Destek: Yazarlar bu çalışma için herhangi bir mali destek almadıklarını beyan etmişlerdir.

Hayvan Refahı: Yazarlar, araştırmalarda kullanılan hayvanları korumak amacıyla ARRIVE Kılavuzlarını izlediklerini beyan ederler.

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