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*In the Name of God, the Compassionate, the Merciful*

# Iranian Journal *of* Veterinary Science and Technology




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## SCOPE

**Iranian Journal of Veterinary Science and Technology (IJVST)** publishes important research advances in veterinary medicine and subject areas relevant to veterinary medicine including anatomy, physiology, pharmacology, bacteriology, biochemistry, biotechnology, food hygiene, public health, immunology, molecular biology, parasitology, pathology, virology, large and small animal medicine, poultry diseases, diseases of equine species, and aquaculture. Articles can comprise research findings in basic sciences, as well as applied veterinary findings and experimental studies and their impact on diagnosis, treatment, and prevention of diseases. IJVST publishes four kinds of manuscripts: Research Article, Review Article, Short Communication, and Case Report.

## ON THE COVER

Posterior spiracles of *Oestrus ovis;* the third instar larva is up to 20 mm long and yellow in colour when young, changing to light brown later and in the maturing stage it shows broad transverse blackish bands dorsally. The second segment is provided dorsally with a variable number of small denticles, the following segments are bare, but provided with a rough leather-like skin pattern, distinct only on the darkened parts. Ventrally the segments bear rows of strong spines. Posterior spiracles in instar III are D shape with a central button without distinct suture; *see page 7.*

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Iranian Journal of Veterinary
Science and Technology

Editor- in- Cheif Lecture

## Editor- in- Cheif Lecture

I have many thanks to the merciful God for publishing the first issue of the Iranian Journal of Veterinary Science and Technology under the new Editor-in-Chief in 2023. For this reason, I will share a few lines with the audience of the Journal.

First, I would like to express my gratitude to the scientific community of veterinary medicine in and out of Iran, who have helped the editors of the journal to provide better service with their scientific support. It is also a great opportunity for all the management members of the journal to express their gratitude to the previous Editors-in-Chief, especially Dr. Hesam Dehghani. During his astonishing seven-year management from 2017 to 2023, the journal reached a prestigious position at the international level and got the Scopus index as well as other databases. Moreover, IJVST was approved and recognized as the journal of the highest ranking by the Ministry of Science, Research, and Technology of Iran in 2020. The page layout of articles was improved to the level of international standards, and the issues were published on time. The publication timeline of the journal was changed from "Semiannual" to "Quarterly" and the journal possesses a logo by his attempts.

Now, I have the great honor of being the Editor-in-Chief and accept the challenges and responsibilities of developing IJVST. Moreover, it is my great honor to work with an exceptional team of editorial board members who generously devote their time and expertise to the journal.

From the very beginning, I considered it necessary to improve the previous achievements of the journal and keep pace with the progress of veterinary science around the globe. My goals as the new Editor-in-Chief are recruiting additional editors and adjusting the expertise of the editorial board as the journal grows. We would also like to take this opportunity to invite researchers worldwide to submit their enlightening research output as research papers and reviews to this journal. We wish to improve the quality of the published papers, acquire the WOS index and other reliable scientific databases, and maintain the speed of review and publication, which has been suitable, except for the recent issue which had a delay due to the changes in management.

I will welcome and value greatly your suggestions and ideas for making the journal highly impactful. Together, with your support, we can build a journal of excellence to serve the research community.

Mehrdad Mohri, DVM, DVSc.
Editor-in-Chief
IJVST

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# Morphometric and genetic characterization of *Oestrus ovis* in sheep in different climatic regions of Iran

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Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

## ABSTRACT

Oestrosis is nasal myiasis, which results from infestation with the larvae of flies that belong to the genus *Oestrus*. Nasal discharge and sneezing are the most common clinical signs in infected animals. Myiasis larvae were collected from sheep in different climatic regions of Iran. Morphological identification of the larvae was made based on the diagnostic keys. The species was confirmed by PCR amplifying the partial fragment (610 bp) of the mtCO1 gene. Genetic distance was assessed in CO1 sequences, and a phylogenetic tree was drawn. Sequencing showed no difference in the partial CO1 gene among the Iranian isolates, and this gene had a high similarity with the sequences of *O. ovis* isolates from Iraq, Bosnia and Herzegovina, and Croatia. The present study provided the first molecular dataset for *O. ovis* species, which is crucial for the phylogenetic relationships assessment and the molecular identification of these parasites.

**Keywords**

*Oestrus ovis, Sheep, CO1, PCR, Phylogeny, Sequence*

Number of Figures: 6
Number of Tables: 1
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## Abbreviations

*CO1: Cytochrome oxidase I*
*PCR: Polymerase chain reaction*
*mtCO1: mitochondrial cytochrome oxidase subunit I*
*MBST: Molecular Biological System Transfer*



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## Introduction

Oestrosis is nasal myiasis, resulting from infestation with the larvae of flies belonging to the genus *Oestrus* (Diptera: Oestridae). It is regarded as a severe parasitosis in goats and sheep and sometimes in other animal species [1]. Nasal discharge and sneezing are the most common clinical signs in animals [2]. Parasitic larvae seriously affect the host's well-being and cause the loss of milk, meat, and wool [3]. The sheep bot fly (*Oestrus* ovis L.) causes severe cavitary myiasis in all areas worldwide where goats and sheep are reared [4]. O. *ovis* can be diagnosed by the morphological characterization of the larvae (e.g., the slits of the posterior spiracles located on the posterior spiracular plates), clinical presentation, and infrequent adult fly identification [5]. A few years ago, molecular techniques were extensively used to achieve insights into the systematics and taxonomy of various insects. Particularly, hypervariable regions within ribosomal and mitochondrial DNA are robust genetic markers to tackle taxonomic diagnostic issues for some flies that cause myiasis and belong to the Oestridae family [6,8]. The mtCO1 gene was chosen to characterize the Oestridae larval relationships. It has previously been revealed to be necessary for various molecular phylogenetic targets due to its comparatively enormous size and the inclusion of both highly conserved and variable regions with different mutational rates [9]. The CO1 658 bp region is a standard and broadly considered animal taxa universal marker [10]. Over the past decade, the barcoding of DNA has provided an effective instrument for phylogenetic characterization using CO1 sequences and molecular identification of various insect species comprising the Oestridae family [11,14]. Therefore, the current study aimed to utilize PCR and partial mtCO1 gene sequencing as a diagnostic tool to identify and demonstrate the phylogenies of larvae in different climatic regions of Iran. The molecular detection of O. ovis is substantial to address its epidemiology and control.

## Results

### *Morphological identification*

The identification of the mature larvae of O. ovis was based on morphological characteristics. The third instar larva is up to 20 mm long, yellowish-white, and tapering anteriorly. Every fragment has an obscure lateral band on the dorsal side. They have a big black oral hook linked to the internal cephalopharyngeal skeleton. The ventral surface is lined with small spines. The results corresponded with what is mentioned in the identification key [15].

### *PCR amplification and sequencing of mtCOI gene*

Figure 1 shows that the isolates in this study had a similar sequence alignment and they all are placed in the same consensus modification group. The 610 bp fragment of mtCO1 from all isolates was successfully amplified (Figure 1). The 610 bp CO1 region covered by the consensus sequences from all reads was effectively acquired with high-quality chromatogram scores. The sequence of the amplified fragments revealed a genome arrangement typical of the O. ovis sequence in GenBank. Phylogenetic tree elucidated phylogenetic relationships between other members of the family Oestridae published in GenBank and O. ovis larvae amplicons as shown in Figure 3. The mean reported interspecific similarity value of CO1s in the Oestrinae subfamily was 86.7% [16]. However, the sequence indicated a 99% identity using the CO1 sequences of O. ovis available at GenBank and < 91% identity using the CO1 sequences of other Oestrus species (Oestrus sp.) [17]. Furthermore, this sequence identity using the CO1 sequences of other Oestrid species was < 90%. These findings established that the examined larvae belonged to O. ovis.

Sequencing results of Iranian O. ovis isolates from six sheep sinuses in this study showed no difference in partial CO1 genes among six Iranian isolates, and they had identical gene sequences (Figure 3). The CO1 gene sequences from the other mitochondrial genomes available in GenBank were chosen and aligned. The obtained sequences in this study (MZ972997 - MZ973002) were compared with GenBank sequences.

Figure 2 demonstrates the comparison of the isolated sequences in this study with sequences in the gene bank, which shows the differences and similarities in the alignment process. Based on multiple nucleotide sequence alignments (Figure 2) and sequence identity matrix algorithms, the percent identities among O. ovis isolates in this study were 100% with O. ovis from Iraq (MW555828), Bosnia and Herzegovina (MG755264), and Croatia (MN845130), and 99% with O. ovis from Iran (KX268655), Spain (MW145179), and Brazil (KR820703). The nucleotide differences with other isolates are exhibited in Figure 3. As a result, a phylogenetic tree was inferred with CO1 gene sequences from this study and previously published correlated species of mitochondrial sequences. The phylogenetic reconstruction showed two main branches (A and B) and a sub-branch (A1) that contained our isolate (MZ972997 - MZ973002) and homolog sequences with high similarity from Iraq, Bosnia and Herzegovina, and Croatia (Figure 3).

About 100% similarity of Iranian sheep isolates with isolates from Iraq, Bosnia and Herzegovina, and

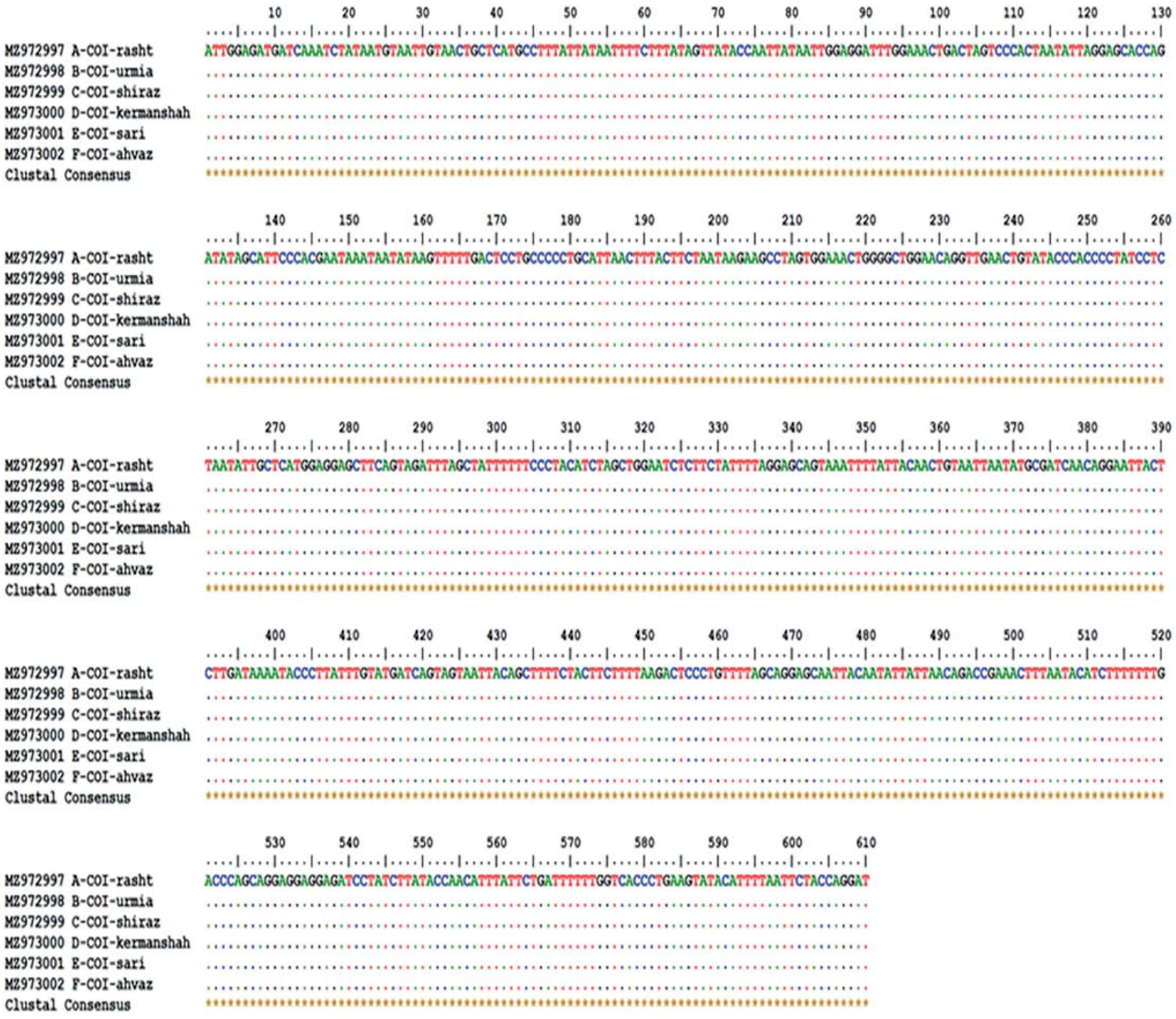


**Figure 1.**
Nucleotide alignment of the Oestrus ovis partial COI sequences of Iranian isolates

Croatia, shows a very close relationship, and homology between the nucleotide sequences of O. ovis isolates from sheep sinuses mtCO1 gene in these geographical areas. Figure 4 shows the alignment process between the isolates of this study and the isolates of the same genus and species identified from the GenBank.

## Discussion

This study analyzed the genetic diversity of O. ovis based on mtCO1 gene nucleotide sequences isolated from the sinuses of Iranian sheep. The mtDNA provides more accurate identifications of the species to which larvae belong, and even closely related species can have different developmental characteristics [18, 19]. Phylogenetic analysis demonstrated that none of the sheep sequences obtained in this study had intraspecific variations and high similarity with the sequences of O. ovis isolates from sheep mtCO1 gene from Iraq, Bosnia and Herzegovina, Croatia, Brazil, and Spain (Figure 3).

The results of the nucleotide alignment of MZ972997 - MZ973002 with other sequences revealed that variation in the A chain of the phylogenetic tree between O. ovis sequences was in position 258 (T > C) for KR820703, MW145179, KX268655, and MT124626, 372 (G > A) for MT124626, 435 (T > A) for MW555828, 456 (C > A) for MT124626, and 459 (T > C) for MW145179, MN845130, MG755264, and MT124626 (Figure 4). The genes encoding proteins in the mtDNA are more diverse than the ribosomal genes [20]. Based on molecular alignment and phylogenetic tree analysis in this study, the close relationship of Iranian isolates with Iraq, Bosnia and Herzegovina, and Croatia from the same hosts was maybe correlated with the same geographical conditions and the high rate of trade between countries [21, 22]. Furthermore, the results of this study showed that all

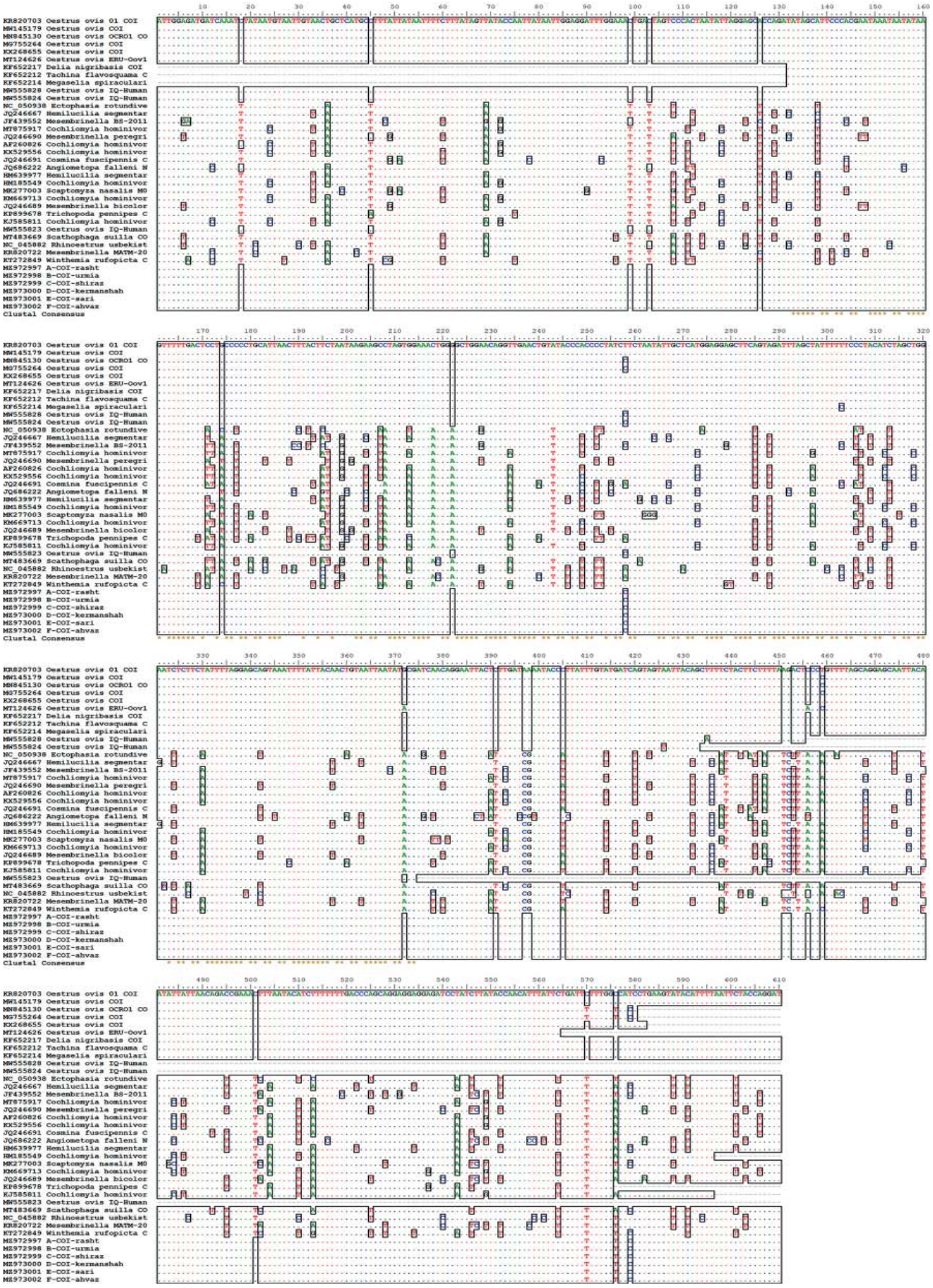


**Figure 2.**
Nucleotide alignment of the Oestrus ovis partial CO1 sequences of Iranian isolates and GenBank

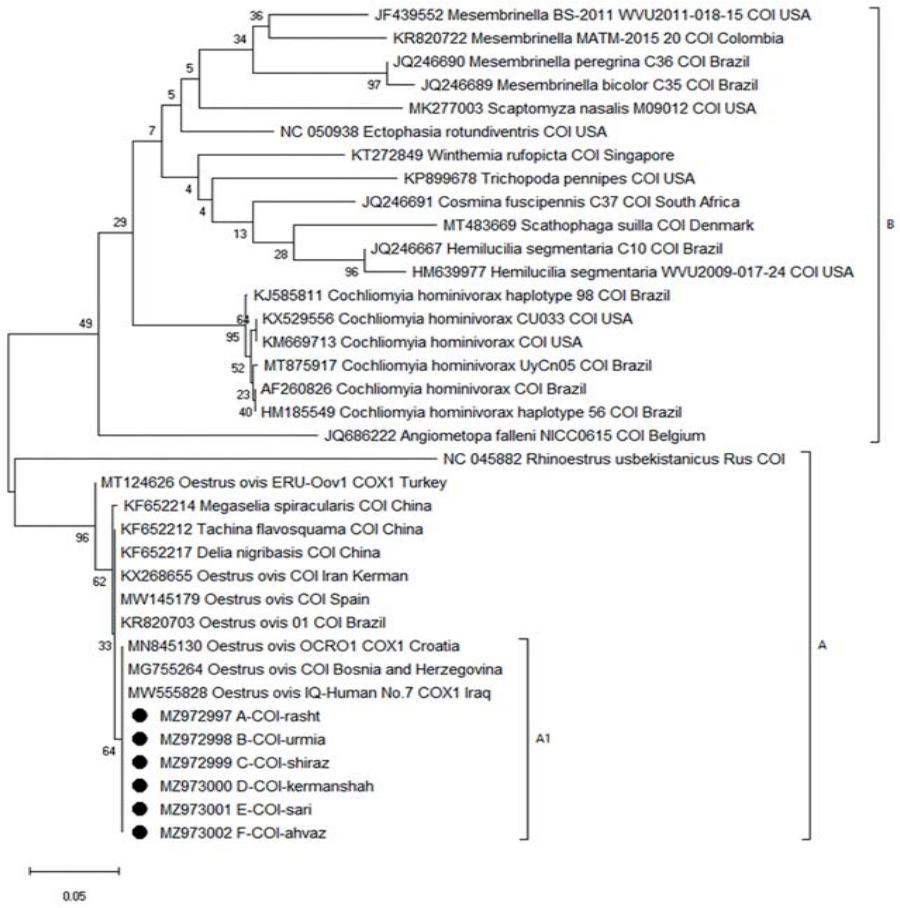


**Figure 3.**
Neighbor-Joining phylogenetic tree of Oestrus ovis partial CO1 sequences

O. ovis obtained in this research and from GenBank were grouped into a monophyletic group according to the results of the previous work [23, 25]. Molecular studies are being used to investigate taxonomy and perform substantial status for identifying Oestridae larvae that lead to obligate myiasis [26].

The physical traits of the larvae and morphological identification are unreliable [27]. Therefore, the molecular identification of myiasis-causing fly species may be considered a credible substitutional to morphological identification as it is challenging to recognize larvae to the genus level [27, 29]. In this study, the high similarity between the isolates from Iran and other countries isolated from the same host and interestingly between other species from other hosts were observed. The results revealed that the genetic variability of O. ovis isolated from different geographical areas of Iran was not clear based on a 610 bp fragment of the mtCO1 gene. Although a low rate of intraspecific variation existed in O. ovis, especially in different hosts, more O. ovis isolates should be collected for further investigations, and complete gene sequences from isolates would be required. The differences between O. ovis isolates may result from variations in the animal breed, geographical area, and climate [22, 24, 25, 31, 32].

Bosly, in 2018, reported a molecular identification of O. ovis larvae, which was recognized as a reliable replacement for morphological identification [25]. This research gives the initial molecular depiction data on O. ovis larvae in sheep in Iran using the barcode CO1 sequences. Our results showed a close association between O. ovis from different countries, even though limited sequences were included in the dataset. The barcode CO1 sequence successfully analyses the species of the subfamilies in Oestriade, and this result shows the usefulness of the CO1 as a suitable diagnostic molecular marker for identifying and characterizing bot fly species.

## Materials & Methods

### *Collection and identification of larvae*

O. ovis larvae were collected from the sinuses of infected sheep in the slaughterhouse of three climatic regions of Iran, including the coastal areas of the Caspian Sea, mountain plateau areas, and flat plains around the Persian Gulf. Two provinces were selected from each region, and ten samples were collected from each of these provinces. Collected larvae were conserved in 70% C2H5OH for morphometric identification and molecular survey. The larvae were identified based on their morphological traits and the three slits at the posterior larvae's spiracle (Figure 5) [15, 33].

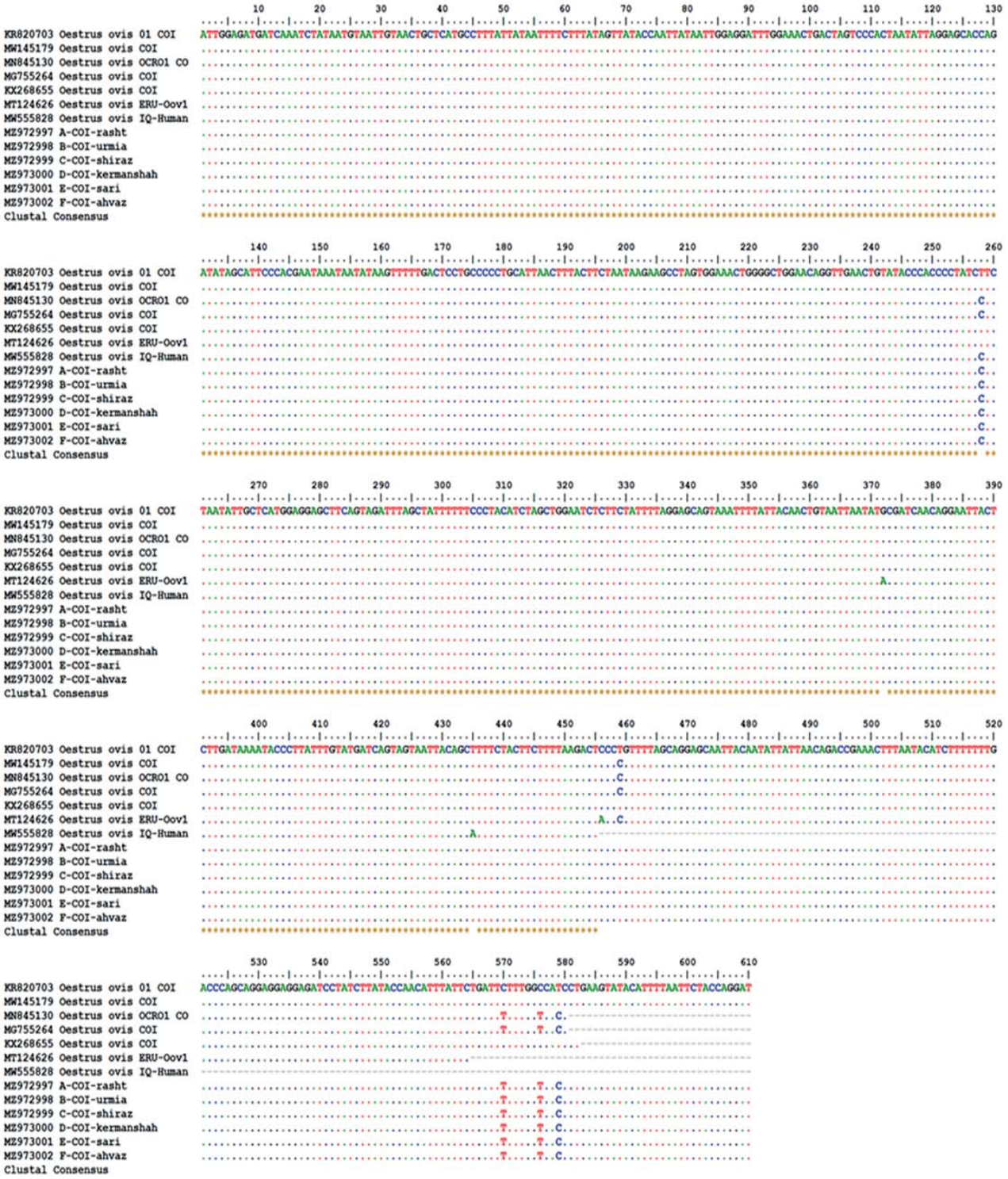


**Figure 4.**
Nucleotide alignment of the Oestrus ovis partial CO1 sequences of Iranian isolates and Oestrus ovis partial CO1 of GenBank

## *Morphological identification and microscopic examination*

The mature larvae of O. ovis were identified based on the morphological characteristics of the larval stages (Figure 6). The third instar larva is 20 mm long and yellow when young, changing to light brown later, and in the maturing stage finally, bands are appear on the dorsal of the third-stage larva.It shows wide transverse blackish bands dorsally. The second segment has a variable number of small teeth on the dorsal side with the rest of the segments being bare. Ventrally, the segments bear rows of strong spines (Figures 6A and B). The findings corresponded with what was mentioned in the identification key [15].

## *DNA extraction*

DNA was extracted from larvae using a DNA isolation kit (MBST, Tehran, Iran) according to the manufacturer's instructions.

**Figure 5.**
Posterior spiracles of Oestrus ovis

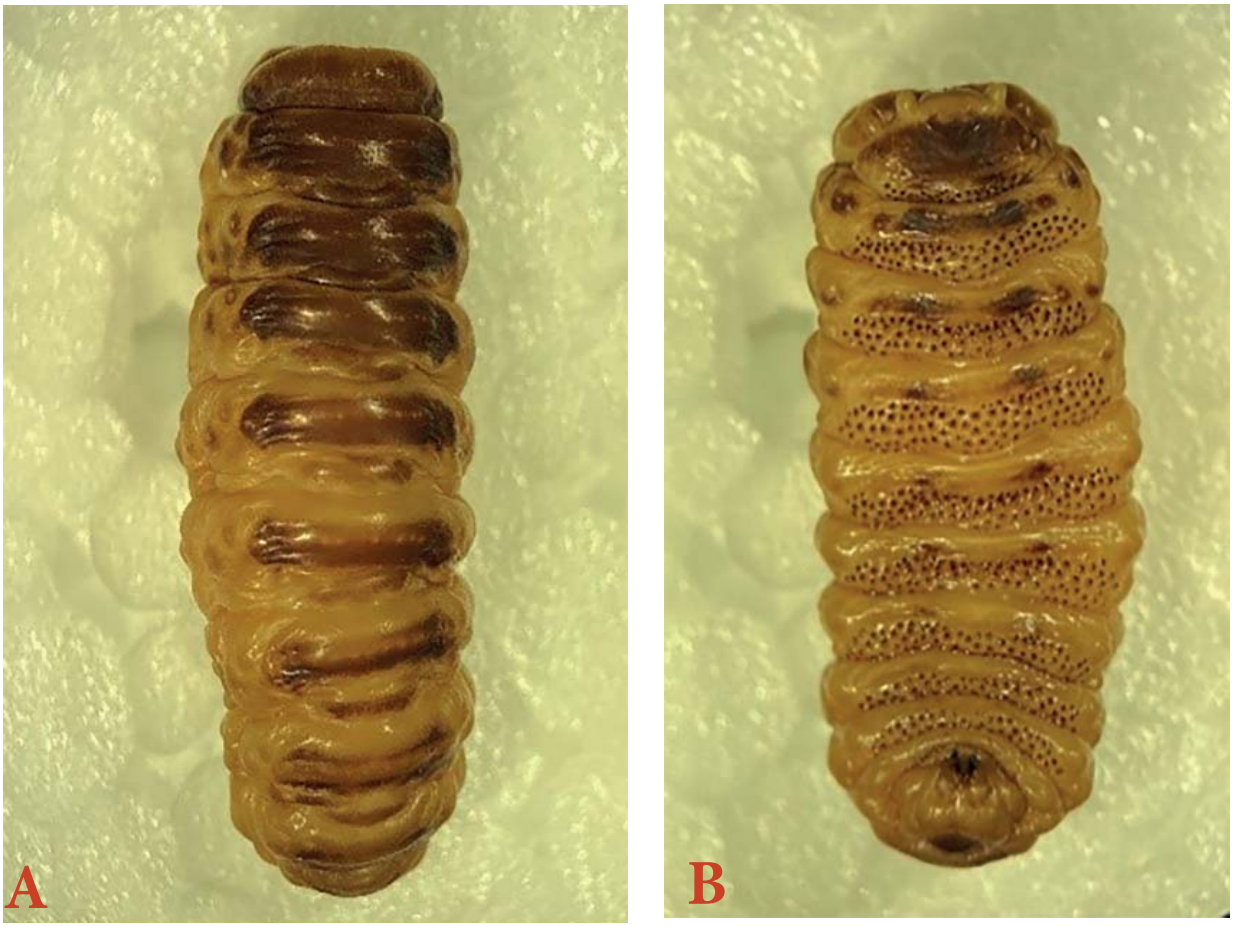


**Figure 6.**
Mature third instar larva of the sheep bot fly Oestrus ovis; A: Dorsal and B: Ventral view

### *PCR procedures and sequencing*

Extracted DNA from each sample was used for amplifying a 610 bp fragment of the mtCOI gene barcoding region using the FFCOI forward (5'-GGAGCATTAATYG-GRGAYG-3') (34) and R-HCO reverse (5'- TAAACTTCAGGGTGACCAAAAAT-CA-3') primers (35). The PCR reaction was a total of 25 µl containing 1 µl of DNA template, 0.25 mM dNTPs, 1x enzyme buffer, 0.25 mM MgCl2, 2.5 µM primers (FFCOI / R-HCO), and 0.5 U of Taq DNA polymerase. The PCR conditions were 40 cycles of initial denaturation at 94°C for 30 sec, followed by denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec. At the final extension step, the reaction terminated at 72°C for 10 min. During PCR, control reactions were performed without adding DNA to the reaction to exclude contamination. To assess the amplified fragment, the amplicons from the PCR were electrophoresed on 1.5% agarose gel and were compared with a marker of standard molecular weight. All amplicons were sequenced using an ABI-3730XL capillary machine (Macrogen Inc., South Korea). O. ovis COI nucleotide sequences were trimmed for any error in nucleotides and the primer sequences were removed using DNA SISMax 3.0 software and then, were submitted to GenBank (Table 1). Based on nucleotide sequences, Bioedit 7.0 software was used for multiple sequence alignments with the Clustal W method. Using the Neighbor-Joining method, phylogenetic trees, and statistical analyses were conducted. Moreover, the evolutionary distances were computed utilizing the p-distance method with the MEGA X software. The analysis involved 36 nucleotide sequences with several 1000 bootstrap replications. Sequences were compared with the identities of isolates confirmed using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were retrieved from GenBank and were utilized to paradigm a phylogenetic tree.

**Table 1.**
*Oestrus ovis* partial CO1 sequences originated within different geographical regions used in this study

| Isolate | Country-Provenience | Host | Nucleotide accession number |
|---|---|---|---|
| A-CO1 | Iran- Rasht | Sheep | MZ972997 |
| B-CO1 | Iran-Urmia | Sheep | MZ972998 |
| C-CO1 | Iran-Shiraz | Sheep | MZ972999 |
| D-CO1 | Iran-Kermanshah | Sheep | MZ973000 |
| E-CO1 | Iran-Sari | Sheep | MZ973001 |
| F-CO1 | Iran-Ahvaz | Sheep | MZ973002 |

## Authors' Contributions

M.T. designed the study. B.E. helped for designing the study and writing of manuscript. S.R. sampling and laboratory examination. All authors reviewed the manuscript.

## Acknowledgements

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## Conflict of interest

The authors declare that they have no competing interests.

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# Distribution of antimicrobial resistance and some widespread extended-spectrum beta-lactamase genes in different phylogroups of Shiga toxin-producing *Escherichia coli* (STEC) isolates of ruminant origin

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## ABSTRACT

Limited data is available on the prevalence of ESBL genes in the STEC isolates of ruminant origin. This study investigated the molecular prevalence of ESBL-encoding genes ($bla_{CTX-M}$, $bla_{TEM}$, $bla_{SHV}$ and $bla_{OXA}$) and AMR of 58 STEC isolates recovered from cattle (n = 32), sheep and goats (n = 26). In the current study, ESBL genes were identified by the molecular technique. Moreover phenotypic AMR was tested by disc diffusion method against six antibiotics , namely amoxicillin-clavulanic acid, tetracycline, neomycin, florfenicol, enrofloxacin, and sulfamethoxazole-trimethoprim. Phylogenetic groups were also determined by a PCR scheme. Isolates were categorized into five phylogroups of (A, B1, C, D, and E), with B1 being the most prevalent phylogenetic group (43; 74.1%). Statistical analysis revealed a significant association between phylogroup D and small ruminants (sheep and goats, $p = 0.014$). Moreover, the highest rates of antimicrobial resistance were related to tetracycline (25.9%) and neomycin (22.4%). Isolates resistant to tetracycline ($p = 0.001$), trimethoprim-sulfamethoxazole ($p = 0.013$) and neomycin ($p = 0.00$) were significantly prevalent among strains recovered from cattle. In addition, the majority of multidrug-resistant strains also had a significant distribution among cattle isolates ($p = 0.001$). In the current study, the prevalence of ESBL positive STEC was 12.06% (7/58). Genes $bla_{CTX-M}$ and $bla_{TEM}$ were detected separately and in combination in bovine isolates. However, only one STEC strain of small ruminants harbored $bla_{TEM}$. In conclusion, it seems that cattle isolates are notable sources of different AMR traits which could be a threat to veterinary sections, public health and food hygiene, in particular.

**Keywords**

*E. coli, STEC, antimicrobial resistance (AMR), ESBL, $bla_{CTX-M}$, $bla_{TEM}$, Phylogroup.*

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## Abbreviations

*ESBL: extended-spectrum beta-lactamase*
*STEC: Shiga toxin-producing Escherichia coli*
*AMR: antimicrobial-resistance*
*HC: hemorrhagic colitis*
*HUS: hemolytic uremic syndrome*



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## Introduction

Human disease caused by STEC range from mild diarrhea to HC and potentially life-threatening HUS [1]. The STEC was the third most prevalent foodborne pathogens in the European Union which increased during the last decade [2]. Ruminants, particularly cattle, have been identified as the most major STEC reservoir. Furthermore, sheep and goats also play a key role in the spread of STECs into the food chain [3]. The STEC has also been isolated from wild animals, and has been reported as a safety risk in the production of fresh fruits and vegetables [4].

Antimicrobial misuse in animal production systems has sent a warning signal to the world's public health. This event resulted in the evolution of antibiotic-resistant strains, and it was with estimated that AMR caused disease mortality to rise from 700,000 in 2014 to 10 million by 2050 [5]. The AMR is regarded as a severe problem in healthcare settings because its mobile genetic elements can alter antibiotic resistance patterns in pathogenic and commensal *E. coli*, as well as the intestinal microbiota of animals and humans [6]. In contrast, little is known about AMR in STEC, particularly when it comes to broad-spectrum beta-lactamases, such as ESBLs from the $bla_{CTX-M}$, $bla_{TEM}$, $bla_{OXA}$, and $bla_{SHV}$ families of ESBL variations [7]. Another issue with resistant-STEC is the spread of these ESBL-coding genes across other *Enterobacterales,* endowing them with antibiotic resistance [8]. Although the use of antibiotics to treat STEC infections is still controversial, antibiotics given early in the course of the infection may help to avoid HUS according to some studies [9]. In this scenario, the frequency of resistant-STEC strains is concern since disease progression continues unabated. The STEC has a high level of genomic plasticity, with mobile genetic components including plasmids, bacteriophages, and genomic islands playing a key role in the transmission of genes, particularly those involved in virulence [10].

The role of genetic background in antibiotic resistance has also been widely investigated in *E. coli*, but to the best of our knowledge has not been studied in ESBL genes among STEC. Based on some previous research, certain members of phylogenetic groups A and D are prone to acquire resistance against third-generation cephalosporins, while B2 strains are more vulnerable [11]. A multiplex PCR, which can classify *E. coli* isolates into eight phylogenetic groups, is the most practical approach for identifying phylogroups A, B1, C, E, D, F, B2, and E. Clades. *E. coli* strains are not randomly dispersed among bacterial populations. Therefore, phylotyping is a useful tool in different genotyping studies. Pathogenicity, niche, and resistance features of the members of the same group tend to be similar [12]. In the present study, we evaluated the STEC isolates of ruminant origin (sheep, goats, and cattle) to build a clear picture of status of AMR, and some important resistance genes in 58 STEC isolates recovered in recent years. Results would help combat AMR in both veterinary and public health sections.

## Results

### *Phylogenetic groups*

We classified 58 STEC isolates into five phylogroups (A, B1, C, D, and E) according to Clermont's phylogrouping method. Members of groups A, B1, and C were identified among all the sources, while group D was only related to sheep and goats (5/26) and E was only detected in cattle (1/32). Moreover, group B1 was the most prevalent phylogenetic group in all sources (sheep and goats: 17/27, 29.3%; cattle: 26/32, 44.8%) and overall (43/58; 74.1%). Statistical analysis revealed a significant association between phylogroup D and small ruminants ($p = 0.014$). No other notable relations were observed. The results are represented in details in table 1.

### *Antibiotic resistance*

**Phenotypic resistance:**

A total of 58 isolates were investigated for phenotypic resistance to six different antibiotics, including: tetracycline (TET), trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanic acid (AMC), neomycin (NEO), florfenicol (FLO) and enrofloxacin (ENFX), by disk diffusion method. The highest rates of antimi-

**Table 1.**
Distribution of STEC isolates in five phylogenetic groups.

| Source (n) | Phylogenetic groups | | | | |
|---|---|---|---|---|---|
| | A | B1 | C | D | E |
| **Cattle (32)** | 4 (6.9%) | 26 (44.8%) | 1 (1.7%) | 0 | 1 (1.7%) |
| **Sheep / Goats (26)** | 2 (3.4%) | 17 (29.3%) | 2 (3.4%) | 5 (8.6%) | 0 |
| **Total (58)** | 6 (10.3%) | 43 (74.1%) | 3 (5.2%) | 5 (8.6%) | 1 (1.7%) |
| ***p*-value** | 0.681 | 0.231 | 0.582 | **0.014*** | 1.000 |

a. *significant difference ($p < 0.05$).

**Table 2.**
Frequency of phenotypic antimicrobial resistance and ESBL genes of STEC isolates (n, %).

| Source (n) | Antibiotics | | | | | | MDR | ESBL genes | |
|---|---|---|---|---|---|---|---|---|---|
| | TET | SXT | AMC | NEO | FLO | ENFX | | $bla_{CTX-M}$ | $bla_{TEM}$ |
| **Cattle (32)** | 14 (24.1%) | 7 (12.1%) | 3 (5.2%) | 13 (22.4%) | 3 (5.2%) | 0 | 13 (22.4%) | 5 (8.6%) | 2 (3.4%) |
| **Sheep/Goats (26)** | 1 (1.7%) | 0 | 3 (5.2%) | 0 | 0 | 1 (1.7%) | 1 (1.7%) | 0 | 1 (1.7%) |
| **Total (58)** | 15 (25.9%) | 7 (12.1%) | 6 (10.3%) | 13 (22.4%) | 3 (5.2%) | 1 (1.7%) | 14 (24.1%) | 5 (8.6%) | 3 (5.2%) |
| ***p*-value** | **0.001*** | **0.013*** | 1.000 | **0.000*** | 0.245 | 0.448 | **0.001*** | 0.058 | 1.000 |

a.*significant difference ($p < 0.05$)

crobial resistance were related to tetracycline (25.9%) and neomycin (22.4%). Moreover, resistant isolates to tetracycline ($p = 0.001$), trimethoprim-sulfamethoxazole ($p = 0.013$), and neomycin ($p = 0$) were significantly prevalent among strains recovered from cattle. In addition, the majority of MDR strains also had a significant distribution among cattle isolates ($p = 0.001$). Table 2, represents the results in details.

**ESBL/β-Lactamase genes:**

Isolates were screened for four widespread ESBL/β-Lactamase genes, namely $bla_{CTX-M}$, $bla_{TEM}$, $bla_{SHV}$, and $bla_{OXA}$ gene families. The genes $bla_{SHV}$ and $bla_{OXA}$ were absent among the STEC isolates. Moreover, only seven isolates (12.06%) possessed ESBL genes. Among them, one isolate harbored $bla_{CTX-M}$ and $bla_{TEM}$ simultaneously, while the remaining six strains carried only one gene. Furthermore, the gene $bla_{CTX-M}$ was only present in cattle isolates. Figure 1 and table 2, represent the results in details.

**Correlations:**

Correlations between AMR, ESBL genes, and MDR were measured and presented in details in table 3. Notable strong correlations were observed between tetracycline and neomycin with each other, and MDR.

**Distribution of antibiotic- resistant isolates among phylogenetic groups:**

The majority of the isolates resistant to five antibiotics (all the antibiotics except enrofloxacin) and MDR were observed in group B1 as it was the most frequent phylogroup. Interestingly, all the $bla_{CTX-M}$ + and 66.6% of $bla_{TEM}$ + (2/3) strains also belonged to the isolates in phylogroup B1. The rest of the resistant isolates were scattered among groups A, D and E. Group C did not show any phenotypic resistance, while one of the $bla_{TEM}$ + strains was a member of group C. Statistical analysis revealed no significant difference in the distribution of antibiotic resistant isolates between phylogenetic groups; except for enrofloxacin, an important quinolone, which was significantly related to phylogenetic group D. However, based on the scarcity of the group D in our study such a difference cannot be conclusive. The results are represented in details in table 4.

**Table 3.**
Correlations between AMR, ESBL genes, and MDR

| *rho* / p-value | TET | SXT | AMC | NEO | FLO | ENFX | $bla_{CTX-M}$ | $bla_{TEM}$ | MDR |
|---|---|---|---|---|---|---|---|---|---|
| **TET** | - | 0.627* | 0.187 | 0.910* | 0.395* | -0.078 | 0.239 | 0.218 | 0.955* |
| **SXT** | 0.000 | - | -0.126 | 0.689* | 0.630* | -0.049 | 0.075 | -0.087 | 0.657* |
| **AMC** | 0.159 | 0.347 | - | 0.089 | -0.079 | -0.045 | 0.097 | 0.432* | 0.205 |
| **NEO** | 0.000 | 0.000 | 0.507 | - | 0.435* | -0.071 | 0.277* | 0.248 | 0.953* |
| **FLO** | 0.002 | 0.000 | 0.554 | 0.001 | - | -0.031 | 0.206 | -0.055 | 0.414* |
| **ENFX** | 0.559 | 0.715 | 0.737 | 0.595 | 0.818 | - | -0.041 | -0.031 | -0.075 |
| $bla_{CTX-M}$ | 0.070 | 0.577 | 0.467 | 0.035 | 0.121 | 0.762 | - | 0.206 | 0.257 |
| $bla_{TEM}$ | 0.101 | 0.518 | 0.001 | 0.061 | 0.684 | 0.818 | 0.121 | - | 0.232 |
| **MDR** | 0.000 | 0.000 | 0.122 | 0.000 | 0.001 | 0.577 | 0.051 | 0.080 | - |

a.The table simultaneously represents *p*-values (numbers on the left side of the table diameter) and Spearman's correlation coefficients (numbers on the right side of the table diameter); Colored cells: strong correlations ($rho > 0.8$); *: Correlation is significant ($p < 0.05$)

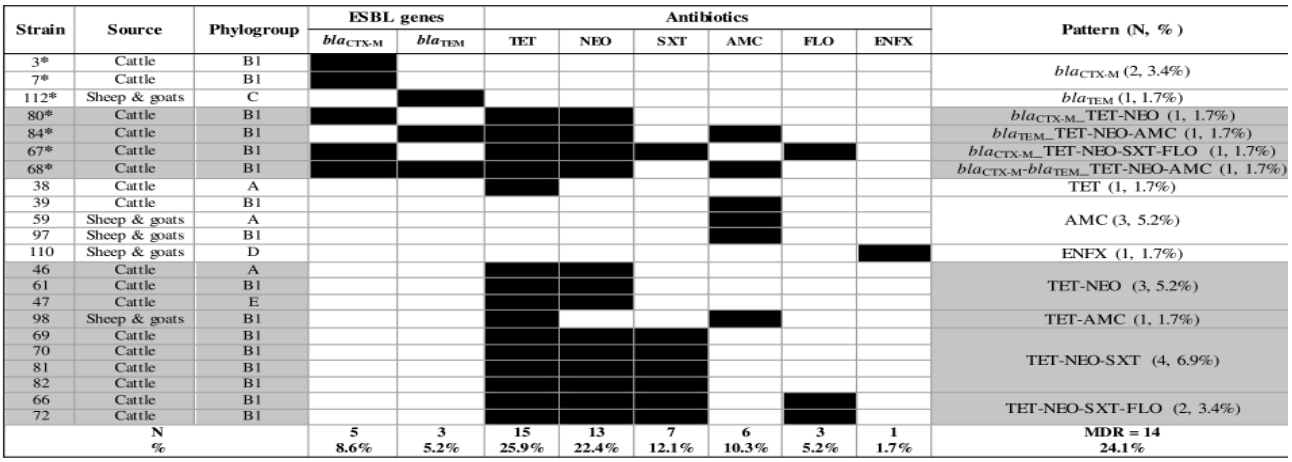


**Figure 1.**
AMR patterns for 22 resistant STEC isolates. Black = having resistance genes/not susceptible; White = no gene/susceptible; Gray = MDR; * = ESBL +

**Table 4.**
Distribution of phenotypic antimicrobial resistance and ESBL genes in STEC belonging to different phylogroups (n, %)

| Phylogroups (n) | Antibiotics | | | | | | MDR | ESBL genes | |
|---|---|---|---|---|---|---|---|---|---|
| | TET | SXT | AMC | NEO | FLO | ENFX | | $bla_{CTX-M}$ | $bla_{TEM}$ |
| **A (6)** | 2 (33.3%) | 0 | 1 (16.7%) | 1 (16.7%) | 0 | 0 | 1 (16.7%) | 0 | 0 |
| **B1 (43)** | 12 (27.9%) | 7 (16.3%) | 5 (11.6%) | 11 (25.6%) | 3 (7%) | 0 | 12 (27.9%) | 5 (11.6%) | 2 (4.7%) |
| **C (3)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (33.3%) |
| **D (5)** | 0 | 0 | 0 | 0 | 0 | 1 (20%) | 0 | 0 | 0 |
| **E (1)** | 1 (100%) | 0 | 0 | 1 (100%) | 0 | 0 | 1 (100%) | 0 | 0 |
| **Total** | 15 | 7 | 6 | 13 | 3 | 1 | 14 | 5 | 3 |
| ***p*-value** | 0.205 | 0.596 | 0.849 | 0.189 | 0.894 | 0.029* | 0.184 | 0.753 | 0.237 |

a.*significant difference ($p < 0.05$)

## Discussion

Today, it is well established that livestock is an important reservoir of pathogenic *E. coli* with public health significance [13]. The presence of STEC strains which are responsible for a wide range of clinical manifestations from mild diarrhea to HC and HUS in humans, has been shown in food-producing animals, especially cattle [14,15]. Emerging AMR in the STEC strains of animal and food sources is a public health threat, as the possibility of resistant genes acquisition by other bacteria is increased [16]. Among different AMR, ESBL has gained a lot of attention during the last decade and ESBL-producing *E. coli* strains have been isolated from livestock as well [17,18]. However, there is a lack of knowledge on the occurrence of ESBL among STEC strains in cattle, sheep and goats as they are one of the main suppliers of milk and meat in most parts of the world. From this perspective, the current study has been conducted to evaluate the prevalence of ESBL-encoding genes among the STEC isolates of ruminant origin.

In the present study, the prevalence of ESBL positive STEC was 12.06% (7/58) which is higher than the reports of Ewers et al., (2/149; 1.34%) and Elmonir *et al.*, (7/100; 7%) [19, 20]. In fact, there is a lack of literature relevant to ESBL in STEC for comparison, as most studies on ESBL-producing E. coli have been performed in the non-STEC isolates of ruminants. Furthermore, bovine is the main subject in such studies, whereas ovine and caprine are mostly neglected. In other words, although livestock is known as STEC reservoirs, only a few studies have addressed the ESBL-producing STEC in cattle, sheep, and goats [21–25]. Moreover, some of the mentioned studies have focused on food hygiene aspects [21,24,26], while attention to gut-isolated pathogens is also valuable, because the intestinal tract is a 'melting pot' and one of the suitable milieu for gene exchange among bacterial

species [27].

To date, several types and subtypes of ESBL-encoding genes have been detected in meat, milk and stool samples of ruminants. For example, ESBL genes such as $bla_{CMY}$ [24,28], $bla_{TEM}$ [18,23,24,26,29], $bla_{SHV}$ [18,23,24,26], and ampC [22,24] have been reported in cattle as well as sheep and goats samples, while $bla_{OXA}$ has been only reported from bovine *E. coli* strains [18]. Moreover, combination of $bla_{CTX-M}$ + $bla_{TEM}$ seems to be more common in *E. coli* with animal origin [24,30]. It seems that $bla_{CTX-M}$ is the most prevalent ESBL-encoding gene in both bovine and small ruminant *E. coli* strains [18,21–24,26,28,29]. in the present study, $bla_{CTX-M}$ and $bla_{TEM}$ were detected separately and in combination in bovine isolates, whereas only $bla_{TEM}$ was identified in one strain of STEC of small ruminants. Our finding are in line with the mentioned reports.

In the present study, most of the ESBL-producing strains which were recovered from cattle belonged to group B1 (6/7; 85.71%). It has been shown that the ESBL positive *E. coli* strains of livestock are mostly related to phylogenetic groups A and B1 and a lesser extent are related to B2 and D which is similar to our results [23, 26, 28, 30, 31]. However, one of our isolates (1/7; 14.28%) which was recovered from small ruminants belonged to phylogenetic group C. As shown by Atlaw *et al.*, (2021), the ESBL positive strains of sheep and goats could be rarely scattered among non-commensal groups such as C and E [31].

Although antibiotic therapy in infections caused by STEC is now contraindicated due to the elevated risk of HUS in some cases, research on using antibiotics that inhibits transcription or translation, such as rifamycins (alone or in combination with fluoroquinolones), showed promising results. This, may lead to changes in the treatment regimen using antibiotics in future [32,33]. Currently, the importance of the emergence and spread of AMR in STEC is getting clear. The more resistant traits STEC has, the poorer the response to therapeutic strategies will be. One of the well-known factors in the emergence of AMR in STEC is the extensive use of antibiotics in clinical and agricultural environments. Today, the occurrence and increase of AMR in the STEC of various populations (human, livestock, companion animals,and the environment) have been documented [34,35]. In our research, resistance to tetracycline (25.9%), neomycin (22.4%), trimethoprim-sulfamethoxazole (12.1%) and amoxicillin-clavulanic acid (10.3%) was observed which is in line with other studies that have noted resistance to tetracycline, aminoglycosides, sulfonamides and β-lactams as the most horizontally acquired AMR in STEC [34,35].

Occurrence a positive strong correlation of resistance to tetracycline with MDR is one of the notable observation in the current study. This, can be partially confirmed by the results recorded by Bourely *et al.* (2019), which proposed the resistance to tetracycline and amoxicillin as an indicator for MDR in *E. coli* recovered from animals [36]. We indicated a strong correlation between neomycin resistance and MDR as well. However, there is a lack in literature relevant to this finding to be compared with.

In conclusion, ESBL-encoding STEC strains were detected in cattle, sheep, and goats in the present study. Moreover, bovine strains showed higher AMR in both ESBL positive and ESBL negative STEC isolates which could be due to the extensive application of antibiotics in the cattle industry for therapeutic and non-therapeutic purposes, such as growth promotion [36,37]. As antibiotic use can lead to a pressure for the emergence and spread of AMR, it seems that more caution should be taken in the veterinary field for antibiotic application especially in sections related to cattle.

## Materials & Methods

### *E. coli Isolates*

A panel of 58 non-duplicate archived STEC *E.coli* isolates was chosen from the bacterial collection (Ferdowsi University of Mashhad, Iran), including 32 isolates from cattle, and 26 isolates from sheep and goats. These bacteria were isolated during 2010 - 2018 in the context of different previous studies and surveyed for the presence of *stx* genes. In brief, the original sampling procedure included collecting fecal samples using sterile cotton swabs from the rectum of animals. In cases with a sample transfer time of more than 24 h, Amies (Oxoid, UK) transfer medium was used. The samples were cultured on MacConkey agar (Merck, Germany) and a pure isolate from each sample was confirmed as *E. coli* using standard biochemical tests [38]. All mentioned isolates were cryopreserved as stocks at -70 ˚C and recovered on Brain Heart Infusion broth (Merck, Germany) with the subsequent additional streak on MacConkey agar to confirm the purity.

To confirm the identity of the STEC isolates, a PCR protocol proposed by Lin *et al.*, (1993) was applied based on the recognition of a common sequence of different stx types or subtypes. Each PCR reaction was performed in a volume of 20 µl containing: 10 µl Taq DNA Polymerase Master Mix RED 2x (Amplicon, Denmark) containing 1.5 mM $MgCl_2$, 1 µl of each primers (Macrogen, Seoul, South Korea), ultrapure water and 300 ng of template DNA. Primer characteristics and thermal conditions are shown in Table 5. Finally, PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and Green Viewer safe stain (0.01 v/v) [39].

### *DNA Extraction*

The crude DNA was extracted using a boiling method as described before [40]. In brief, a suspension of three colonies from an overnight culture (18-20 h) was selected and prepared in sterile tubes containing 300 µl of sterile distilled water. The tubes were boiled in a boiling water bath for 10 min and after cooling on ice buckets centrifuged at 800×g for 5 min. The supernatant was used as a DNA template in the PCR.

### *Determination of phylogenetic groups*

Phylogenetic groups of the isolates were investigated using

the updated method developed by Clermont *et al.*, (2013). The method enables an *E. coli* isolate to be assigned to one of the eight phylogroups (A, B1, B2, C, D, E, F, Clade I) and also allows isolates that are the members of other cryptic clades (II - V) of *Escherichia* to be identified. However, some isolates which cannot be categorized as mentioned groups, are known as 'unknown'. The method consists of a primary quadruplex PCR reaction and additional PCR reactions, when necessary [12].

Each PCR reactions was performed in a volume of 20 µl containing: 10 µl Taq DNA Polymerase Master Mix RED 2x (Amplicon, Denmark) containing 1.5 mM $MgCl_2$, various concentrations of each primers (Macrogen, Seoul, South Korea), ultrapure water, and 300 ng of template DNA. Thermal conditions and primer characteristics are shown in Table 5. Finally, PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and Green Viewer safe stain (0.01 v/v).

## Antimicrobial resistance

**Phenotypic resistance:**

Antimicrobial susceptibility was conducted according to the CLSI recommendations using the disc diffusion method [41]. The antibiotics (Padtan Teb, Tehran, Iran) were chosen from six families of widely used antibiotics in humans and/or animals including: amoxicillin-clavulanic acid (AMC, 20/10 µg), tetracycline (TET, 30 µg), and neomycin (NEO, 30 µg), florfenicol (FLO, 30 µg), enrofloxacin (ENFX, 5 µg), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 µg). The isolates that showed resistance against three or more families of antimicrobials were designated as MDR.

**ESBL genes:**

Molecular detection of ESBL-producing *E. coli* was carried out using using a triplex PCR reaction for $bla_{TEM}$, $bla_{SHV}$,and $bla_{OXA}$ and a uniplex PCR for $bla_{CTX-M}$. Each PCR reaction was performed in a volume of 20 µl containing: 10 µl Taq DNA Polymerase Master Mix RED 2x (Amplicon, Denmark) containing 1.5 mM $MgCl_2$, various concentrations of each primers (Macrogen, Seoul, South Korea), ultrapure water and 300 ng of template DNA. Primer characteristics and thermal conditions are shown in Table 5. Finally, PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and Green Viewer safe stain (0.01 v/v).

## Statistical analysis

In addition to the descriptive analysis of the results, possible relationships of genetic criteria (phylogenetic groups and ESBL genes) with phenotypic AMR were assessed by the chi-squared test and Fisher's exact test. Correlation among AMR, ESBL genes and MDR were also measured and represented using Spearman rank-order correlation coefficient (*rho*). For all the analysis, $p < 0.05$ was considered significant. Moreover, correlations with *rho* > 0.8 were categorized as "*strong correlation*". In the present study, the data were analyzed by SPSS version 16.0 (SPSS Inc., Chicago, USA).

**Table 5.**
Primers used in the present study (STEC, Phylogenetic groups and ESBL genes)

| Panel | Primer pair | Sequence (5' to 3') | Annealing temp (°C) | Product size (bp) | Ref. |
|---|---|---|---|---|---|
| **STEC** | *stx* | F: GAACGAAATAATTTATATGT | 43 | 900 | [39] |
| | | R: TTTGATTGTTACAGTCAT | | | |
| **Phylogenetic grouping** | | | | | |
| **Quadruplex** | *chuA* | F: ATGGTACCGGACGAACCAAC | 59 | 288 | [12] |
| | | R: TGCCGCCAGTACCAAAGACA | | | |
| | yjaA | F: CAAACGTGAAGTGTCAGGAG | | **211** | |
| | | R: AATGCGTTCCTCAACCTGTG | | | |
| | TspE4.C2 | F: CACTATTCGTAAGGTCATCC | | **152** | |
| | | R: AGTTTATCGCTGCGGGTCGC | | | |
| | *arpA* | F: AACGCTATTCGCCAGCTTGC | | **400** | |
| | | R: TCTCCCCATACCGTACGCTA | | | |
| **Group E** | *arpA* | F: GATTCCATCTTGTCAAAATATGCC | 57 | **219** | |
| | | R:GAAAAGAAAAAGAATTCCCAAGAG | | | |
| **Group C** | *trpA* | F: AGTTTTATGCCCAGTGCGAG | 59 | **489** | |
| | | R: TCTGCGCCGGTCACGCCC | | | |
| **ESBL genes** | | | | | |
| **Triplex** | $bla_{TEM}$ | F: CATTTCCGTGTCGCCCTTATTC | 57 | **800** | [42] |
| | | R: CGTTCATCCATAGTTGCCTGAC | | | |
| | $bla_{SHV}$ | F: AGCCGCTTGAGCAAATTAAAC | | **713** | |
| | | R: ATCCCGCAGATAAATCACCAC | | | |
| | $bla_{OXA}$ | F: GGCACCAGATTCAACTTTCAAG | | **564** | |
| | | R: GACCCCAAGTTTCCTGTAAGTG | | | |
| **Uniplex** | $bla_{CTX-M}$ | F: ATGTGCAGYACCAGTAARGTKATGGC | 61 | **593** | [43] |
| | | R:TGGGTRAARTARGTSACCAGAAYCAGCGG | | | |

## Authors' Contributions

Conceptualization, G.H. and M.A.; Methodology, Rw.T. and H.K.R.; Software, H.K.R.; Supervision, G.H. and M.A.; Writing – original draft, R.T. and H.K.R.; Writing – review & editing, Rw.T., H.K.R., G.H., R.T. and M.A. All authors have read and agreed to the published version of the manuscript.

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## Competing Interests

The authors declare that there is no conflict of interest.

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# Radiographic and histological evaluations of the effects of meloxicam and flunixin meglumine on the repair of radial bone defects in a rabbit model

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## ABSTRACT

The current study radiographically and histologically evaluated the effects of meloxicam and flunixin meglumine on the repair of radial bone defects in a rabbit model. Ninety New Zealand White rabbits (10-12 months, 1.5-2.6 kg) were randomly assigned into three groups. Following anesthesia, defects were created on the medial surface of the radius bone of the left forelimb with a diameter and depth of 3 mm. The animals were administrated meloxicam, flunixin meglumine, and physiological serum (positive control) subcutaneously each day for 10 days. Hematoxylin and Eosin and Goldner's trichrome stainings, along with radiograph images were prepared to investigate the effects of the administered agents. The results did not show callus formation in bone defects on days 3 and 7. Defects were filled in meloxicam and positive control groups on day 14, while they were filled on day 21 in the flunixin meglumine group. On days 14 and 21, the meloxicam group outperformed the flunixin meglumine group in terms of callus formation, but it was higher in the flunixin meglumine group on day 28. It could be concluded that the administration of meloxicam is less effective in delaying the bone healing process.

**Keywords**

*Flunixin meglumine, Histopathological, Meloxicam, Rabbit, Radiographic*

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## Abbreviations

*NSAID: Non-steroidal anti-inflammatory drug*
*COX-1: Cyclooxygenase-1*
*COX-2: Cyclooxygenase-2*
*PGE2: Prostaglandins E2*



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## Introduction

*B*one fractures and segmental bone defects are major sources of patient morbidity and costs to the healthcare system [1]. Infection, tumor, trauma, surgery, and congenital aetiologies are major causes of bone defects [2]. The healing of bone defects is the main challenge for veterinarians and physical medicines [3]. Bone healing is a complex biological and biomechanical process and is characterized by three partially overlapping phases, namely the inflammatory phase, repair phase, and remodeling phase [4]. Inflammation plays a pivotal role in the bone healing process and immune-inflammatory modulation is a major challenge for treatment [5]. The NSAIDs, such as meloxicam and flunixin meglumine, are administrated for the management of pain and inflammation in humans and animals. Meloxicam is a selective blocker of COX-2 that is used for managing rheumatoid arthritis, acute exacerbations of osteoarthritis, ankylosing spondylitis, skeletomuscular pains, and juvenile idiopathic arthritis [6]. It mostly exhibits its effects via COX-2 rather than COX-1 [7] and prevents COX-2 which results in inhibiting the conversion of arachidonic acid into pro-inflammatory prostaglandins [8]. Flunixin meglumine is another NSAID agent that is administrated to treat pain and signs of endotoxemia via the inhibition of COX isoenzymes [9]. It also reduces PGE2 concentrations in tissues [10]. Studies have investigated the effects of NSAIDs on the treatment of fractures and reported that NSAIDs have negative effects on mesenchymal stem cells due to the prevention of cell proliferation[11]. Another study showed that the administration of NSAIDs modulated the expression of osteogenic and chondrogenic marker genes [12]. Studies have also shown that NSAIDs may hinder bone healing and increase the risk of other complications [13]. Moreover, it was reported that NSAIDs did not change the proliferation and differentiation of osteoblasts but reduced the activity of plasminogen activators, metalloproteinases, and cathepsin B [14]. Regarding to the effects of NSAIDs on the bone healing process, the effects of meloxicam and flunixin meglumine on the bone healing process have not been still elucidated.

This study was conducted to compare and evaluate the effects of meloxicam and flunixin meglumine on the repair of radial bone defects in a rabbit model using H&E and Goldner's trichrome staining and radiograph images.

## Results

Age and weight differences between the meloxicam, flunixin meglumine, and positive control groups were not found to be statistically significant ($p > 0.05$). All the rabbits underwent anesthesia and surgery and survived until the end of the study.

### *Radiographic images*

Figure 1 depicts the results of radiographic images. Our findings did not show significant differences on days 3 and 7. On the same days, no callus formation was observed. The filling of defects started on days 14 and 21 in the meloxicam and flunixin meglumine groups, respectively. Inflammation was similar between all the groups on the same days. Callus formation was significantly higher on day 28 compared to days 14 and 21 ($p < 0.05$). The findings indicated that the rabbits in the meloxicam group had more calli than those in the flunixin meglumine group on days 14 and 21. However, the score was significantly higher in the flunixin meglumine group on day 28 than in other groups. Furthermore, the scores were higher in the meloxicam group than in the flunixin meglumine group from day 28 until the end of study. There were significant differences between groups from day 42. Table 1 illustrates the scores for the bone healing process.

### *Histopathological results*

Figure 2 shows histopathological results in different groups. The results of the first day in all groups revealed that the defect area was inflamed due to blood clot formation and increased growth of blood vessels. In the first week, blood clots were still visible in the defect area in all groups and collagen was gradually replacing blood clots. Blood clot and collagen ratios were 80% and 20% in the saline group, 85% and 15% in the flunixin meglumine group, and 95% and 5% in the meloxicam group, respectively. In the second week, clots were replaced with collagen and fibrotic tissue. The ratio of collagen to clot was 70% to 30%, 80% to 20%, and 90% to 10% in the positive control, flunixin meglumine, and meloxicam groups, respectively. In the third week, the clots were not seen in defects in control and meloxicam groups and they were also completely replaced with collagen. A small amount of clot (10%) was observed in the flunixin meglumine group. In the fourth week, the results showed that collagen was slightly replaced with cartilage in the control and flunixin meglumine groups, but no cartilage was observed in the meloxicam group. In the fifth week, the rabbits in the control groups had more cartilage. We observed that 30% of the samples had only collagen tissue, while collagen and cartilage tissue were equally apparent in 40% of the samples as the restoration process advanced. In addition, 30% of the samples had cartilaginous tissue, which was greater than their fibrous tissue. The difference between samples in NASID groups with collagen tissue and those who had progressed in their healing was 40%. In

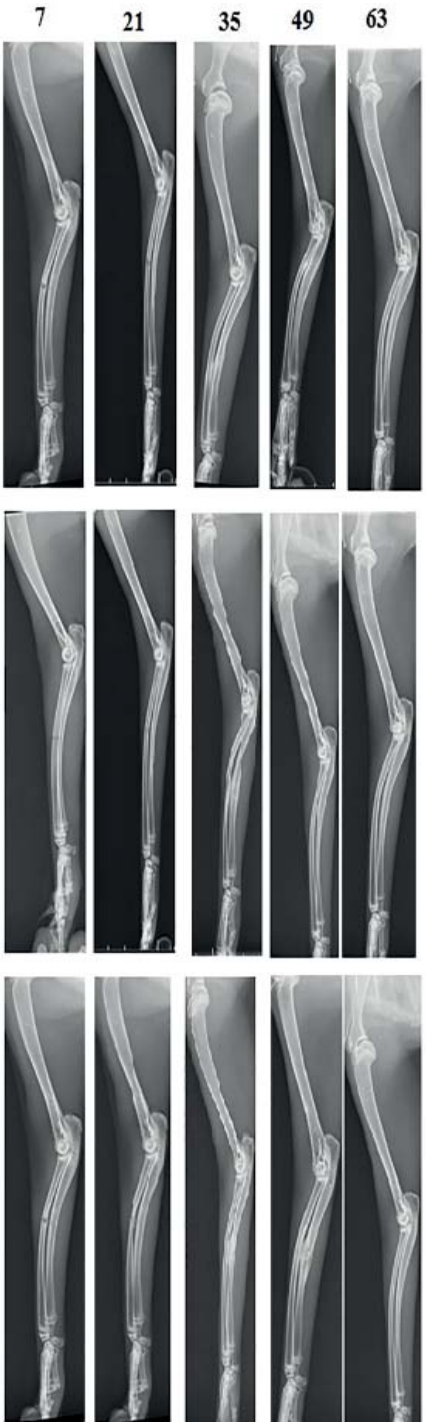


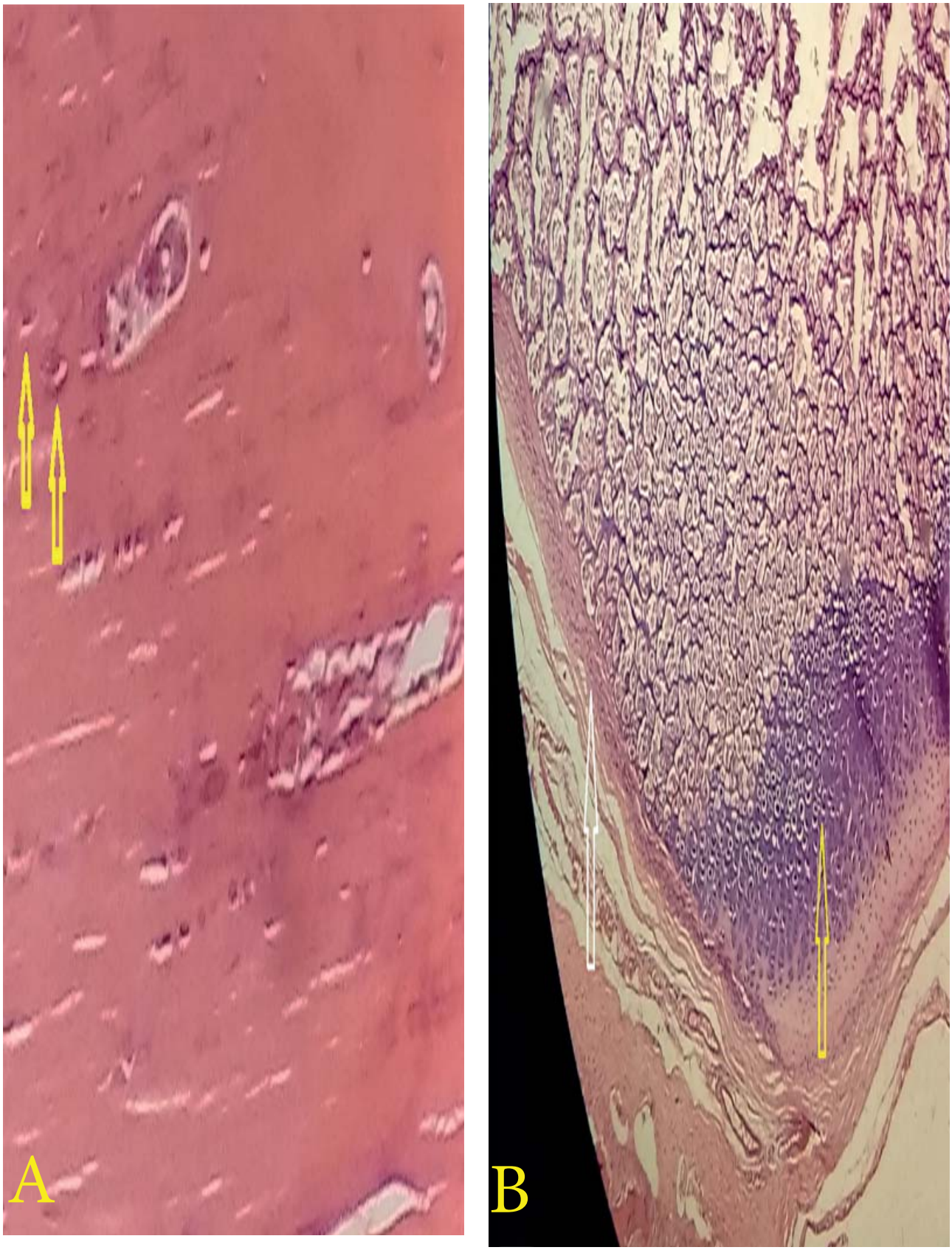

**Figure 1.**
Radiograph images in different groups on days 7, 21, 35, 49, and 63

the 6th week, fibrotic tissue was completely replaced with cartilage tissue in some positive control samples (20%). In the NSAID groups, fibrotic tissue was completely replaced with cartilage tissue in some samples (10%) and cartilage tissue was significantly higher in the meloxicam group. In the 7th week, defects were significantly filled with cartilage in more samples in the control group and fibrotic tissue was completely replaced with cartilage tissue in some samples (10%) and cartilage tissue was significantly higher in the meloxicam group. The results of week 8 indicated that blood clots were not observed in the defect area in all groups and a significant amount of cartilage had replaced the clots. Consequently, there was more progress in the repair. The samples in which the amount of cartilaginous tissue was more than fibrous tissue were measured to be 20%. In addition to cartilaginous tissue, the samples also contained bone tissue, albeit in smaller amounts. In the flunixin meglumine group, samples were counted as having 30% more cartilaginous tissue than fibrous tissue. Moreover, in certain samples (40% of the subjects), the fibrotic tissue had vanished and the defects were cartilaginous. There was less bone tissue than cartilage tissue in 30% of the cases. Samples from the meloxicam group were measured to have 30% more cartilaginous tissue than fibrous tissue. In the 8th week's samples, it was noted that the defect area was completely cartilaginous, and 30% of the fibrotic tissue had vanished. In this eighth week, there were samples that contained bone tissue. However, the amount of bone tissue was low compared to cartilage tissue. These samples made up 40% of the total. The results of week 9 revealed that in 20% of the control group samples, there was more cartilaginous tissue than fibrous tissue, while the remaining samples had an equal quantity of cartilage and bone tissue. We observed that 20% of the samples in the flunixin meglumine group only had cartilage tissue, 40% of the samples had less bone tissue than cartilage tissue, and the remaining samples showed an equal distribution of cartilage and bone tissue. In the meloxicam group, bone tissue was found in 50% of the subjects in lower amounts than cartilage tissue. In addition, bone and cartilage tissue were found in similar amounts in the remaining samples.

**Table 1.**
The scores for the bone healing process

| Groups | 0 | 7 | 14 | 21 | 28 | 35 | 42 | 49 | 63 |
|---|---|---|---|---|---|---|---|---|---|
| **Control** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.85 ± 0.24 | 0.92 ± 0.18 | 1.42 ± 0.44 | 2.00 ± 0.00 | 2.57 ± 0.34 | 2.71 ± 0.26 | 2.64 ± 0.24 |
| **Flunixin M** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.64 ± 0.37 | 1.07 ± 0.18 | 1.14 ± 0.24 | 1.28 ± 0.26 | 1.42 ± 0.26 | 1.57 ± 0.34 |
| **Meloxicam** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.71 ± 0.39 | 0.78 ± 0.26 | 0.85 ± 0.24 | 1.28 ± 0.35 | 1.78 ± 0.26 | 1.88 ± 0.26 | 1.78 ± 0.26 |
| **P-values*** | 1.00 | 1.00 | 0.00 | 0.0231 | 0.012 | 0.0214 | 0.021 | 0.023 | 0.015 |

a.* Statistically significant ($p < 0.05$).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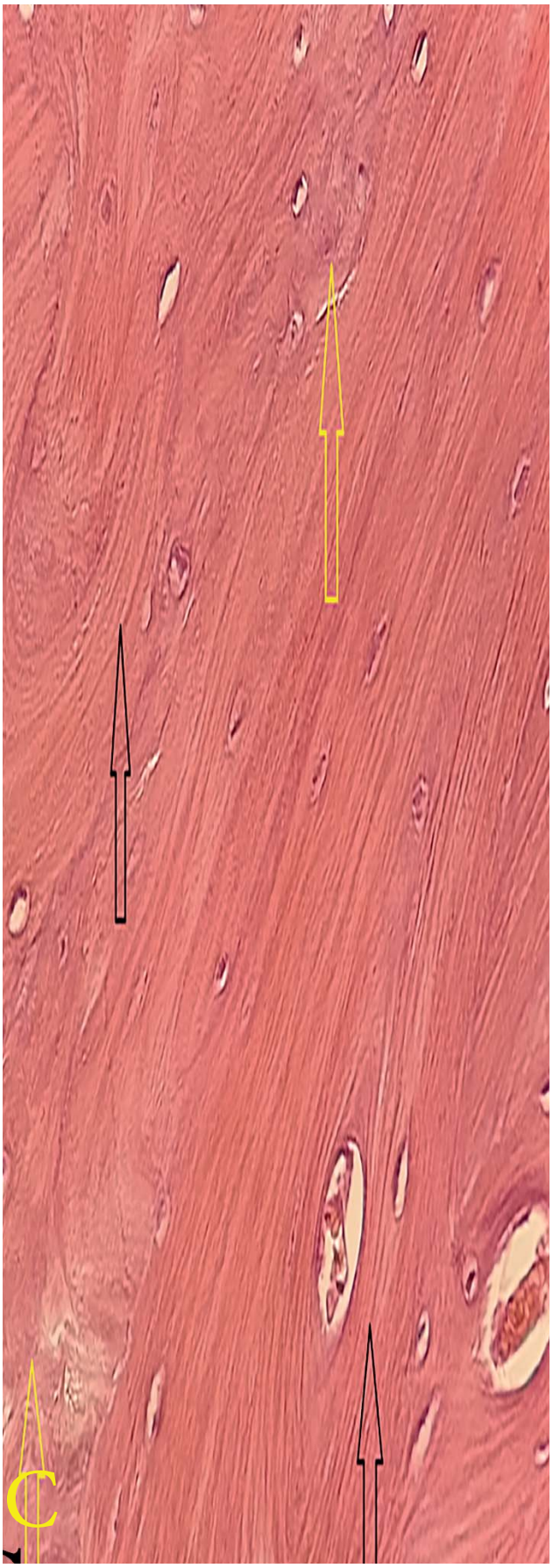
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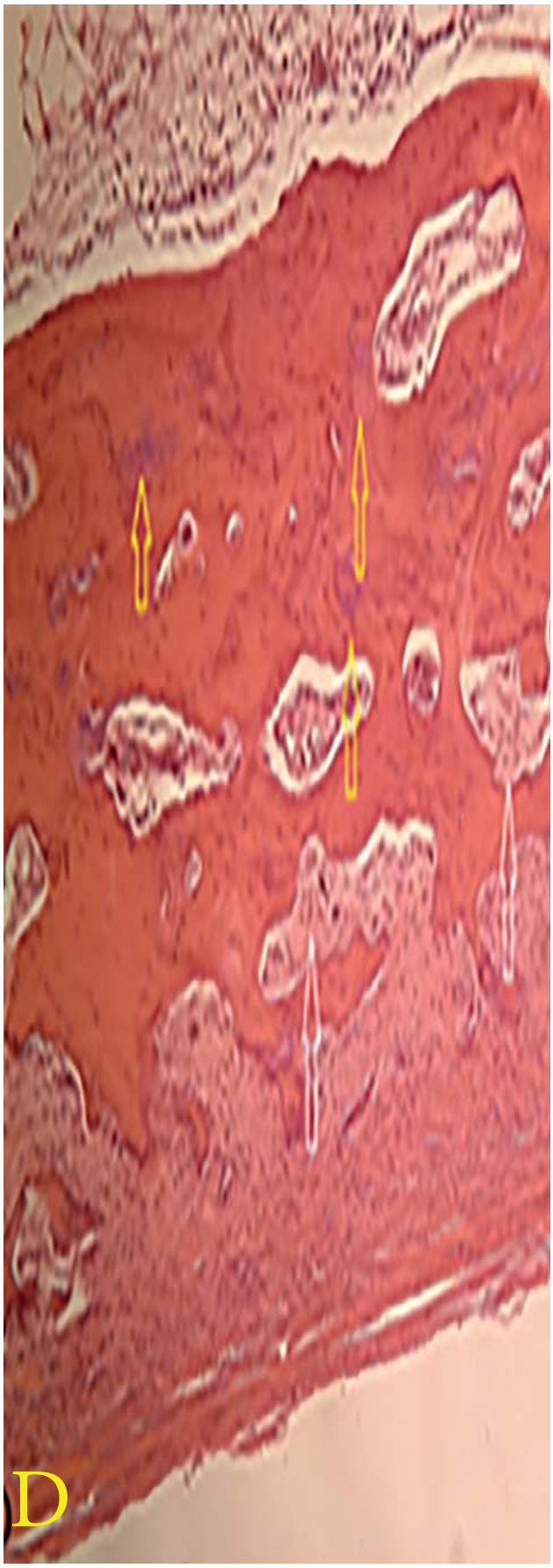
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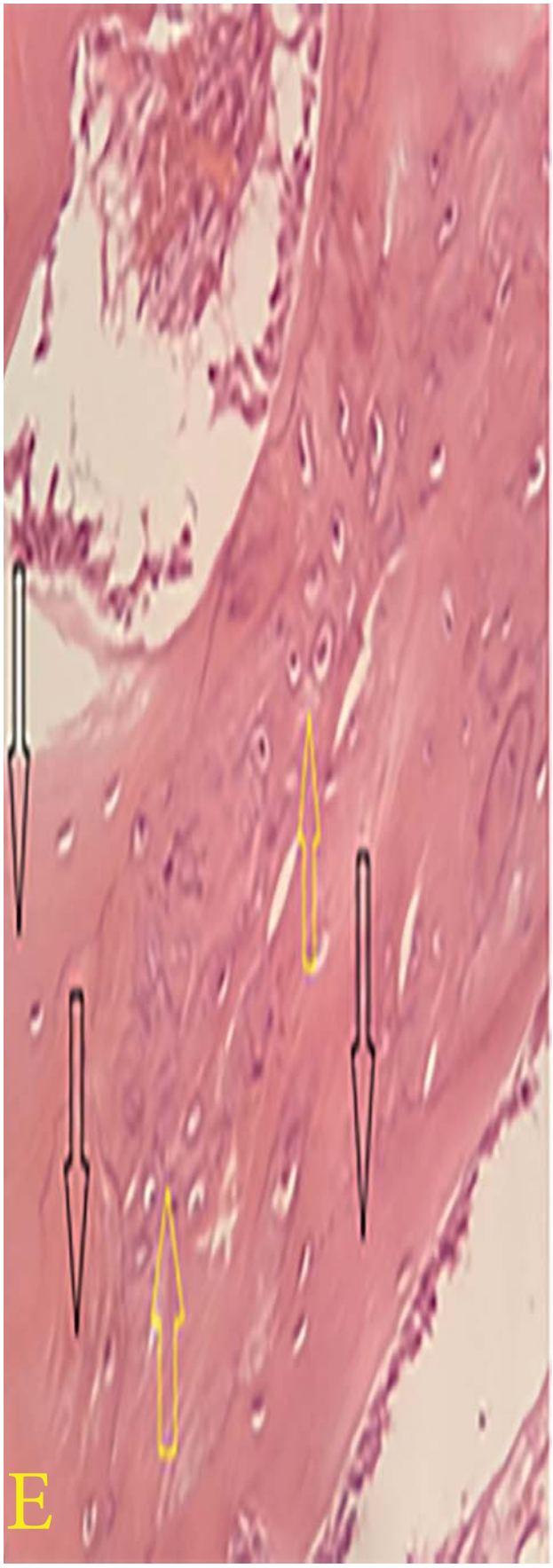
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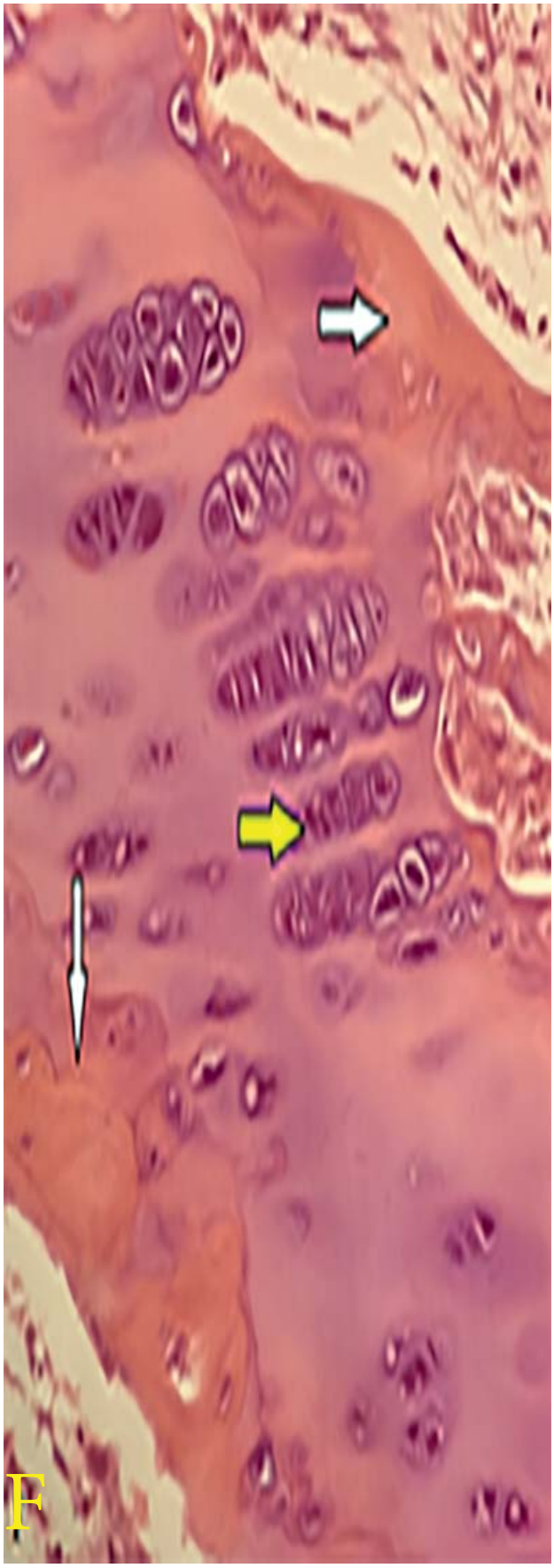
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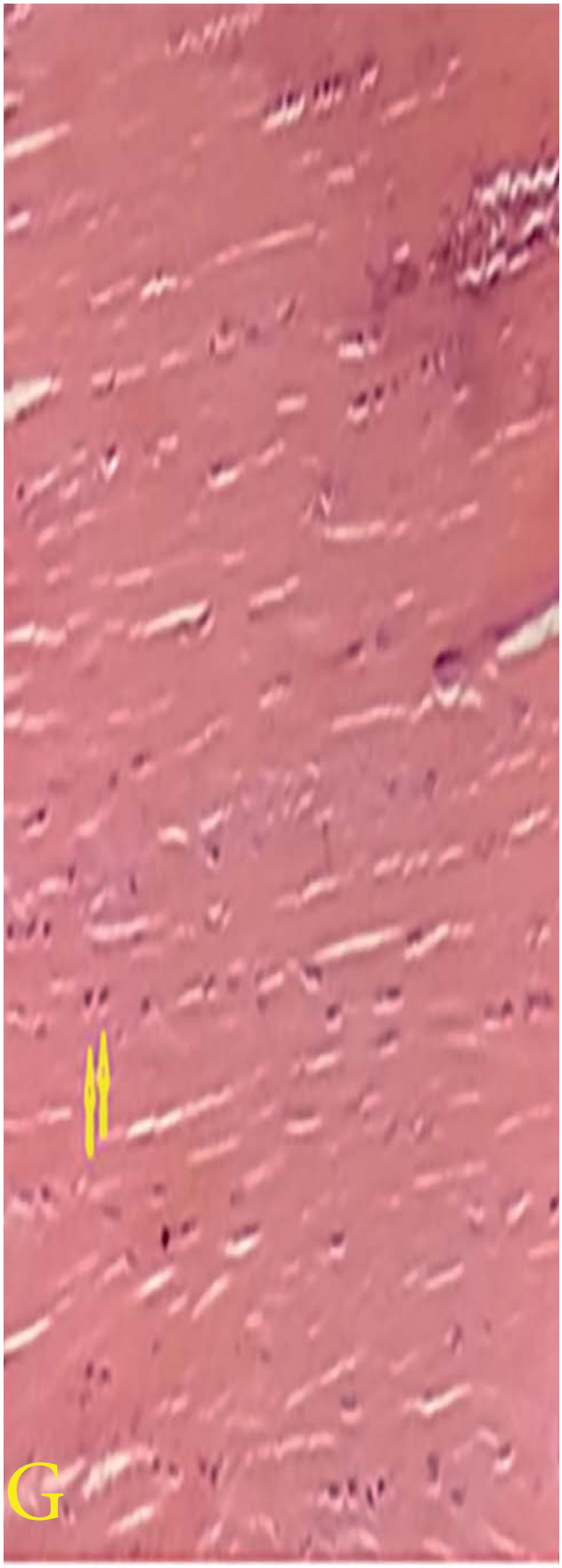
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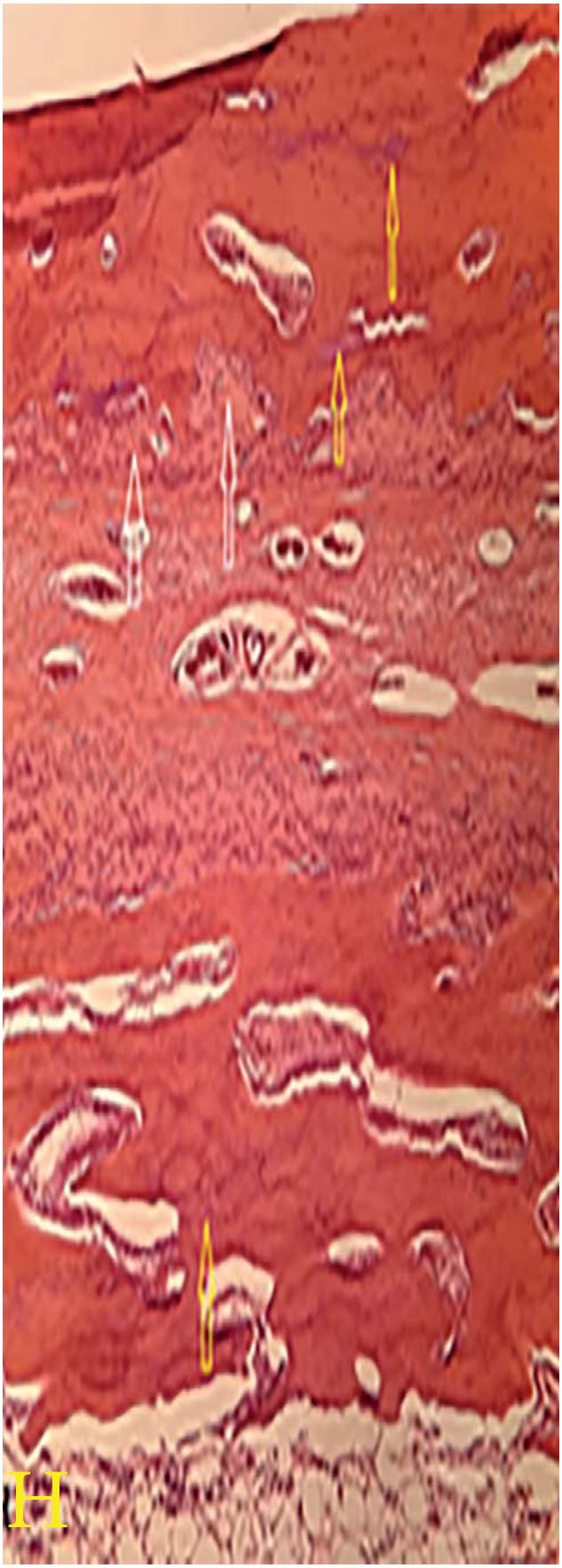
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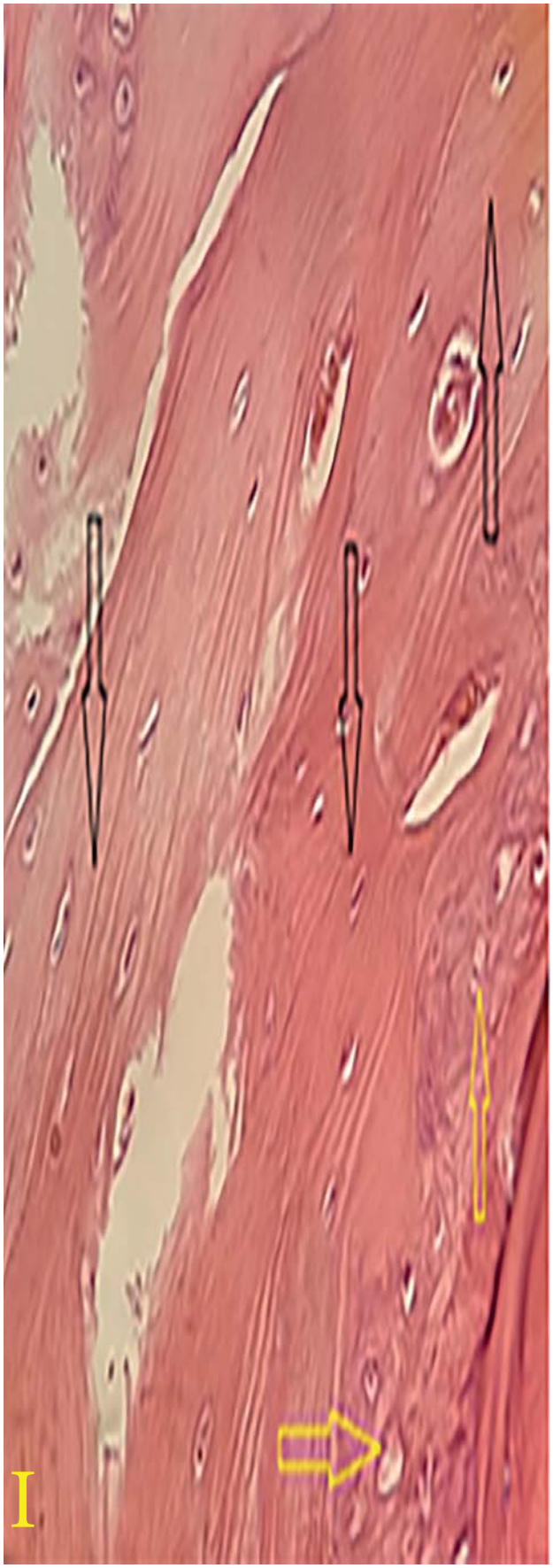


**Figure 2.**
A: Longitudinal section of the radius in rabbits in the third week and collagen is observable. Arrows show fibroblasts. B and C: Transversal section of the defect in the control group. B: There are similar ratios of fibrosis and cartilage tissues in the fifth week. Yellow and white arrows show cartilage and fibrotic tissues, respectively. C: Cartilage tissue is low and bone tissue is significantly found in the ninth week. Yellow and black arrows show cartilage and bone tissues. D-F: Transversal section of defects in the flunixin meglumine group. D: Fibrosis tissue is less and cartilage tissue is significantly observed in the seventh week. Yellow and white arrows show cartilage and fibrotic tissues, respectively. E & F: Cartilage tissue is much and bone tissue is slightly observed in the eighth and ninth weeks. Yellow and black arrows show cartilage and bone tissues, respectively. G-I: Transversal section of the defect in the meloxicam group. G: Fibrosis tissue (collagen) is observable in the fourth week. Arrows show fibroblasts. H: Cartilage tissue is much and fibrotic tissue is too low in the eighth week. I: There is a similar ratio of cartilage and bone in the ninth week. Yellow and black arrows show cartilage and bone tissues, respectively.

## Discussion

This study aimed to evaluate radiographically and histologically the effects of meloxicam and flunixin meglumine on the repair of radial bone defects in a rabbit model. Results showed that NSAIDs significantly reduced the bone healing process compared to the control group. In the first week, the radiographic results did not show significant differences between groups ($p = 1$). On days 14 and 21, there were no significant differences between the meloxicam and the control groups. However, the bone healing process was significantly higher in the control group compared to other groups, indicating that meloxicam and flunixin meglumine did not cause any delay in the bone healing process in the first week compared to the control group. On the other hand, the administration of these agents delayed the bone healing process from the fourth week until the end of the study.

Our results are in line with previous studies that showed NASIDs retard the bone healing process [11-14]. A delay in the bone healing process could be attributed to the effects of NSAIDs on PGE2 because bone and collagen formation is dependent on PGE2 [10]. Prostaglandins have pivotal roles in the regeneration of pericytes in injured bones and stimulate bone maturation. The results showed that the effects of meloxicam on the bone healing process were weaker compared with flunixin meglumine. It means that meloxicam has fewer adverse effects on the bone healing process due to its mechanism of action. Meloxicam primarily demonstrates its effects via inhibiting COX-2 [7] and preventing the conversion of arachidonic acid into pro-inflammatory prostaglandins [8], while flunixin meglumine exerts effects via inhibiting COX isoenzymes [10] and reducing PGE2 concentrations in tissues [14]. Flunixin meglumine appears to involve more COX isoenzymes than meloxicam and to have more potent effects. Histopathological results showed the presence of inflammation in the defects. According to the histopathological findings, the defects had inflammation which is a protective response to tissue trauma and/or a stimulus for removing harmful stimuli and starting the healing process [15]. It is argued that chronic sustained inflammation delays the healing process [16, 17]. The bone healing process might not be accompanied by inflammatory responses and may last for several months [18, 19]. Inflammation is the first step in bone healing and its prevention can expedite the healing process [16, 17]. The NASIDs can reduce inflammation and accelerate the bone healing process. Our findings revealed that the administration of NSAIDs reduced inflammation. A reduction in inflammation may hasten the bone healing process, although NSAIDs inhibit it via other mechanisms. These agents inhibit the production of PGE2 and bone regeneration via interaction with bone morphogenetic proteins [20, 21]. In addition to preventing DNA synthesis and reducing osteoblast proliferation, NSAIDs have no appreciable effects on bone mineralization [22]. Histopathological results indicated that the healing process slowed down in the second week and there was less fibrosis tissue formation in the NSAID groups than in the control group.

The results showed that the formation of cartilage and bone tissues was slower in the NASID groups. Their actions on COX-2 may be responsible for the reduction in bone and cartilage production [6]. It can be stated that inflammation is essential for the bone healing process but it should rapidly occur. The NASIDs inhibit inflammation and slow down bone healing. Our findings showed that NSAIDs inhibit the development of cartilage, collagen, and bone tissues and produce persistent clots over longer periods than in control groups.

It was concluded that NASIDs inhibit callus formation in the defects. Histopathological findings confirmed that NSAIDs prevent callus formation, fibrosis, and bone tissue formation in defects. Flunixin meglumine had greater effects than meloxicam. It is suggested to administer the NASIDs with lower side effects for decreasing pain during the bone fraction

## Materials & Methods

### *Materials*

Meloxicam (Boehringer-Ingelheim; Mobic, Germany), flunixin meglumine (Banamine injectable solution; Merck Animal Health, Madison, NJ, USA), ketamine (Alfasan; Woerden, Netherlands), and xylazine (Bayer; Leverkusen; Germany) were purchased.

### *Animals and surgery*

Islamic Azad University, Urmia Branch approved all the procedures used in the current study for the care and treatment of animals (IR.IAU.URMIA.REC.1400.020). All the efforts were made to minimize pain. Ninety male New Zealand White rabbits (10-12 months, 1.5-2.6 kg) were prepared and kept in a room with free access to water and food. They were housed in a room at 24°C with a 12-h light/dark cycle. To induce anesthesia, each animal was intravenously administrated with 2% pentobarbital sodium (1.5 ml/kg). Bone defects were created as reported by other researchers [23]. The animals were intramuscularly administrated with 1 mg/kg of acepromazine and their left forelimbs were shaved from the middle of the arm to the end of the radius. To induce anesthesia, xylazine (8 mg/kg) and ketamine (70 mg/kg) were administered intramuscularly. Following the isolation of bone periosteum and soft tissues, a defect with a diameter and depth of 3 mm was created on the medial surface of the radius bone of the left forelimb of each rabbit. Soft tissue was overlapped on the defect and sutured. Cefazolin was intramuscularly administrated for three days. The animals subcutaneously received 0.5 mg/kg meloxicam, 1 mg/kg flunixin meglumine, and physiological serum (positive control) 12 h/once for six days and then 24 h/once for four days.

### *Radiographic investigations*

Radiographic evaluations were conducted on days 0, 7, 14, 21, 28, 35, 42, 49, and 63 and were scored as follows; 0: complete defect and lack of radiopacity in the defect region, 1: slight increase in radiopacity in the defect region and initiating the filling defects, 2: defect region not clear and an increase in radiopacity, and 3: defect completely filled and/or defect region similar to adjacent bones.

### *Histopathological evaluations*

Rabbits were sedated with ketamine/xylazine and euthanized by intravenous injection of sodium pentobarbital (120 mg/kg) [24]. For histopathological investigation, sections were placed in formalin 10%, transferred to the histology lab, and cut. Next, the samples were dehydrated using degrading alcohol 70°C-100°C. They were placed in paraffin and frozen. The specimens were prepared at a diameter of 5 µm and deparaffinized and were stained with H&E and Goldner's trichrome. The sections were investigated by a light microscope.

### *Data analysis*

The Shapiro-Wilk test was used to determine the normality of data distribution. Using SPSS software (version 21), the data were analyzed by the Mann-Whitney test. $P < 0.05$ was considered significant.

## Authors' Contributions

Conceived and designed the experiments: HD, SS. Performed the experiments: SS. Analyzed the data: HD, SS, MMM, ARB. Research space and equipment: HD, MMM, ARB. Contributed reagents/materials/analysis tools: HD. wrote the paper: SS, HD.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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# Effects of Adding α-tocopherol to Brahman Bull Chilled Semen on Sperm Quality, Lipid Peroxidation, Membrane Integrity, and DNA Integrity

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## ABSTRACT

During storage at low temperatures, the spermatozoa quality changes due to cold shock and free radicals. Diluent supplementation with antioxidants is an effort to maintain the quality of spermatozoa during storage. This study aimed to evaluate the α-tocopherol effect in CEP extender on sperm quality, membrane integrity, and lipid peroxidation during storage at 4°C-5°C. This was a laboratory experiment that compared the use of 2 mM of α-tocopherol in CEP with no addition of α-tocopherol (as control) in five bulls. Semen was collected from Brahman bulls, diluted in CEP with and without α-tocopherol, and stored at 4°C-5°C. Sperm motility and viability were investigated by a light microscope at a ×400 magnification using Eosin-Nigrosin staining. Moreover, membrane integrity was evaluated by lipid peroxidation using the MDA assay and hypoosmotic swelling test. The sperm motility, viability, and membrane integrity were higher in CEP with α-tocopherol. Lipid peroxidation was significantly different between the treatment and control groups. The α-tocopherol supplementation in the diluent CEP could maintain the spermatozoa quality during storage at 4°C-5°C.

## Keywords

*α-tocopherol, Brahman bull, Cauda epididymal plasma, Frozen, Spermatozoa*

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## Abbreviations

*AI: Artificial insemination*
*BSA: Bovine serum albumin*
*CEP: Cauda Epididymal Plasma*
*EDTA: Ethylenediaminetetraacetic acid*
*MDA: Malondialdehyde*
*PCR: Polymerase chain reaction*
*ROS: Reactive oxygen species*
*SNI: Indonesian National Standard*
*TBA: Thiobarbituric acid*



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## Introduction

Some procedure in sperm preservation (storage) is performed at 4°C-5°C and freezing conditions, which affects sperm quality [1, 2]. Therefore, fresh semen must comply with a standard quality to be frozen, such as 70%-80% of motility and 80%-90% of viability [3]. The storage of semen at a temperature of 4°C-5°C can maintain the quality of spermatozoa for up to 2-3 days [4]. Moreover, this method does not depend on the availability of liquid nitrogen as a preservation agent. However, by modifying the diluent media, the storage time can be extended. Several studies showed an extension of storage time by using INRA96 diluent which could maintain the quality of bovine spermatozoa for up to 4 days [5], in commercial diluent Megapor S.L. Spain was able to maintain the quality of boar spermatozoa for up to 10 days [6].

Spermatozoa preservation at low temperatures cannot prevent the presence of free radicals. It reduces the acrosome integrity, motility, plasmalemma function, and viability, and induces chromatin damage [7, 8]. The freezing of spermatozoa preservation has been also reported to decrease spermatozoa motility and viability. The spermatozoa head region surrounding the plasma membrane is most susceptible to gluttony. Spermatozoa heads have been reported to swell more than 50% [9]. Moreover, it triggers spermatozoa of RNA expression before and after freezing on its preservation [10]. Fresh semen commonly contains not only spermatozoa cells but also other cells, such as leukocytes and epithelial cells, before storage. These cells and immature spermatozoa are the sources of ROS [11].

Lipid peroxidation is caused by free radical and spermatozoa damage [12]. Lipids, proteins, and DNA oxidation are usually induced by high ROS levels in cells [2, 13]. There are high lipid membrane phospholipids in spermatozoa that lead to high ROS generation [14-16]. Spermatozoa with damaged and abnormal morphology produce higher ROS than spermatozoa with normal morphology [17]. Dead spermatozoa are the main source of ROS during storage at low temperatures [18, 19].

Antioxidants are required to reduce lipid peroxidation in the extender. Vitamin E (α-tocopherol) is one of the non-enzymatic antioxidants that protects polyunsaturated fatty acids, cell components, or cell membranes from oxidation by free radicals [20].

The addition of α-tocopherol in the semen diluent affects the spermatozoa quality at low-temperature conditions. It is able to maintain motility and protects spermatozoa from damage [21]. A study reported that the addition of α-tocopherol caused a significant change in the diluents of Madura bull spermatozoa [22]. Furthermore, α-tocopherol can maintain the integrity, motility, and viability of the spermatozoa membrane on the Simental bull after storage at 4°C-5°C. High-quality semen has a motility of ≥ 70% and a viability of ≥ 75%. The use of antioxidants in diluents is useful for maintaining spermatozoa quality [23].

The CEP diluent was first developed by Verberckmoes et al. [24] who used it to store bovine semen at a temperature of 4°C-5°C with the constituent components mimicking the physical and chemical conditions in the cauda epididymal plasma of cattle [25]. They modified the concentration of egg yolk and replaced the antibiotic gentamicin with penicillin-streptomycin, and managed to maintain the quality of spermatozoa until day 8. The CEP diluent is also able to maintain the quality of sexed spermatozoa during storage at low temperatures [26]. This study aimed to evaluate the sperm quality, membrane integrity, and DNA integrity of Brahman bull that was stored in CEP with or without α-tocopherol at 4°C-5°C.

## Results

As shown in Table 1, the mean percentage of sperm motility was not significantly different on days 0-3 between the control and treatment groups. However, a significant difference was found in spermatozoa motility in CEP with α-tocopherol compared to without α-tocopherol on days 4-7. Based on our study, sperm motility was better in CEP with α-tocopherol than without α-tocopherol. There were no significant differences ($p > 0.05$) in the viability percentage of sperms from day 0 to 4. However, on days 4-7, the percentage of sperm viability was higher ($p < 0.05$) in diluents with α-tocopherol than without α-tocopherol (Table 1).

### *Membrane Integrity*

The motility and viability of spermatozoa were assessed along with spermatozoa function during storage based on their membrane integrity. The evaluations of membrane integrity percentage are illustrated in Table 2.

There was no significant difference in membrane integrity at the beginning of storage (days 0-4). On the other hand, there was a significant difference in membrane integrity in each treatment after day 5. The best results were in the CEP diluent using α-tocopherol on day 7.

### *Lipid Peroxidation*

The MDA levels were measured to determine the existence of radicals inside and outside the cell. The MDA results can be seen in Table 3.

**Table 1.**
Effects of α-tocopherol addition to cauda epididymal plasma extender on sperm quality (Motility % and Viability %) during seven days (D) of storage at refrigerator temperature (4°C -5°C)

| Treatments | % Motility in days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 |
| CEP without α-tocopherol | 58.83[a] ± 0.84 | 55.83 [a] ± 0.83 | 52.50 [a] ± 1.43 | 48.33 [a] ± 1.66 | 44.17 [b] ± 0.96 | 41.67 [a] ± 1.67 | 38.33 [b] ± 0.85 | 35.00 [b] ± 0.00 |
| CEP + α-tocopherol | 56.67[a] ± 0.83 | 55.00[a] ± 0.00 | 50.00 [ab] ± 0.00 | 50.00 [a] ± 0.00 | 49.17 [a] ± 0.83 | 46.67 [a] ± 1.66 | 45.83 [a] ± 0.83 | 45.00 [a] ± 0.00 |
| | % Viability in days | | | | | | | |
| CEP without α-tocopherol | 80.76[a] ± 1.06 | 70.94 [ab] ± 0.76 | 73.00 [ab] ± 0.59 | 69.54 [a] ± 0.68 | 62.61 [ab] ± 1.53 | 49.46 b ± 2.68 | 47.17 [b] ± 1.78 | 45.50 [b] ± 1.00 |
| CEP + α-tocopherol | 79.2 [a] ± 0.20 | 71.45 [a] ± 0.82 | 69.77 [b] ± 0.77 | 68.37 [ab] ± 0.43 | 64.13 [a] ± 0.31 | 60.58 a ± 1.31 | 58.44 a ± 0.47 | 56.10 a ± 0.11 |

CEP: Cauda epididymal plasma
[a-b] uppercase letters in the same column indicate significant differences based on Student's T-test (α = 5%)

**Table 2.**
Effects of α-tocopherol addition to cauda epididymal plasma extender on membrane integrity % during seven days (D) of storage at refrigerator temperature(4°C-5°C)

| Treatments | Membrane integrity (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 |
| CEP without α-tocopherol (control) | 79.59 [a] ± 1.06 | 72.61 [a] ± 0.72 | 71.76 [a] ± 0.65 | 68.93 [ab] ± 0.35 | 60.86 [ab] ± 1.60 | 48.55 [b] ± 2.57 | 46.41 [b] ± 1.99 | 43.98 [b] ± 1.33 |
| CEP + α-tocopherol | 78.23 [a] ± 0.32 | 70.53 [a] ± 0.99 | 68.84 [ab] ± 0.95 | 67.69 [a] ± 0.35 | 65.92 [a] ± 0.63 | 65.66 [a] ± 1.94 | 60.91 [a] ± 1.02 | 56.29 [a] ± 0.33 |

CEP: Cauda epididymal plasma
[a-b] uppercase letters in the same column indicate significant differences based on Student's T-test (α = 5%)

**Table 3.**
Effects of α-tocopherol addition to cauda epididymal plasma extender on malondialdehyde values during four days (D) of storage at refrigerator temperature (4°C-5°C)

| Treatments | MDA value | | | |
|---|---|---|---|---|
| | D1 | D3 | D5 | D7 |
| CEP without α-tocopherol (control) | 0.222 ± 0.191 [a] | 0.306 ± 0.179 [a] | 0.390 ± 0.055 [a] | 0.613 ± 0.234 [a] |
| CEP + α-tocopherol | 0.116 ± 0.007 [b] | 0.168 ± 0.168 [b] | 0.175 ± 0.018 [b] | 0.182 ± 0.008 [b] |

CEP: Cauda epididymal plasma; MDA: malondialdehyde
a-b uppercase letters in the same column indicate significant differences based on Student's T-test (α=5%)

There were significant differences in MDA levels between treatments. A significant difference was found on days 1-7 of storage. The lowest MDA levels were shown in the α-tocopherol treatment, while the highest MDA levels were observed in the control group (CEP without α-tocopherol).

### *DNA Integrity*

DNA integrity during storage may change due to the presence of free radicals. DNA integrity was assessed by sequencing a gene that controls spermatozoa motility (NAD1-1) (Table 4).

**Table 4.**
Data of NAD-1 sequences

| | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 |
|---|---|---|---|---|---|---|---|---|
| Control Day-1 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| Control Day-3 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| Control Day-5 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| Control Day-7 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| α-tocopherol Day-1 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| α-tocopherol Day-3 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| α-tocopherol Day-5 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |
| α-tocopherol Day-7 | GTTCTAGGCT | ACATACAACT | CCGAAAAGGC | CCAAATGTCG | TAGGCCCATA | TGGCCTACTT | CAACCTATCG | CCGATGCAAT |

| | 90 | 100 | 110 | 120 | 130 | 140 | 150 | 160 |
|---|---|---|---|---|---|---|---|---|
| Control Day-1 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| Control Day-3 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| Control Day-5 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| Control Day-7 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| α-tocopherol Day-1 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| α-tocopherol Day-3 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| α-tocopherol Day-5 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |
| α-tocopherol Day-7 | CAAACTTTTC | ATTAAAGAAC | CACTACGACC | CGCCACATCT | TCAACCTCAA | TATTCATCCT | AGCACCCATT | TTAGCCCTAG |

| | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 |
|---|---|---|---|---|---|---|---|---|
| Control Day-1 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| Control Day-3 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| Control Day-5 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| Control Day-7 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| α-tocopherol Day-1 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| α-tocopherol Day-3 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| α-tocopherol Day-5 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |
| α-tocopherol Day-7 | GCTTAGCCTT | AACCATGTGA | ATCCCCCTCC | CAATACCCTA | CCCTCTTATT | AACATAAATC | TAGGAATCCT | ATTTATACTA |

| | 250 | 260 | 270 | 280 | 290 | 300 | 310 | 320 |
|---|---|---|---|---|---|---|---|---|
| Control Day-1 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| Control Day-3 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| Control Day-5 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| Control Day-7 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| α-tocopherol Day-1 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| α-tocopherol Day-3 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| α-tocopherol Day-5 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |
| α-tocopherol Day-7 | GCCATATCAA | GCCTAGCCGT | ATACTCTATC | CTCTGATCAG | GTTGAGCCTC | CAATTCAAAA | TACGCACTAA | TCGGAGCCCT |

| | 330 | 340 | 350 | 360 | 370 | 380 | 390 | 400 |
|---|---|---|---|---|---|---|---|---|
| Control Day-1 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| Control Day-3 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| Control Day-5 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| Control Day-7 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| α-tocopherol Day-1 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| α-tocopherol Day-3 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| α-tocopherol Day-5 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |
| α-tocopherol Day-7 | ACGAGCAGTA | GCACAAACAA | TCTCATACGA | AGTAACACTA | GCAATTATCC | TATTATCAGT | GCTCCTAATA | AGTGGGTCCT |

**Table 4 - Cont'd**

| | |
|---|---|
| Control Day-1 | TTACCCTCT |
| Control Day-3 | TTACCCTCT |
| Control Day-5 | TTACCCTCT |
| Control Day-7 | TTACCCTCT |
| α-tocopherol Day-1 | TTACCCTCT |
| α-tocopherol Day-3 | TTACCCTCT |
| α-tocopherol Day-5 | TTACCCTCT |
| α-tocopherol Day-7 | TTACCCTCT |

Note: Alignment results for sample

## Discussion

At the beginning of storage, no significant differences were found between the control and treatment groups. It demonstrated that short storage time did not affect the spermatozoa quality. This result also indicated that the dilution process of semen in CEP with and without α-tocopherol was a success. Therefore, it did not cause a significant change in spermatozoa quality. It was also supported by the spermatozoa quality such as motility and viability become slowly.

In the present study, we revealed that the CEP media was very suitable for the storage of bull spermatozoa, although the semen was categorized as rejected semen. The CEP media could maintain the spermatozoa quality from the rejected semen during the storage process at 4°C-5°C. These findings are also in line with Verbeckmoes [24] and Ducha [25, 27, 28]. The CEP media provides chemical and physical conditions to store bulls' spermatozoa. The basic diluent of CEP was developed by imitating the physical and chemical conditions of bovine epididymal plasm which has an osmolarity in the range of 290-300 mosm, contains ionic components Na, K, Ca, Cl, Mg, P, bicarbonate, energy source molecules (i.e., fructose and sorbitol), molecules for pH balance (i.e., tris and citric acid), as well as antibiotics and egg yolk [24, 27]. Another research [24] showed that CEP was able to maintain the quality of bovine spermatozoa for up to 6 days at a temperature of 4°C-5°C with the addition of 20% egg yolk. Furthermore, the replacement of gentamicin with penicillin-streptomycin showed that CEP could maintain the quality of bovine spermatozoa for up to 8 days [25].

The addition of α-tocopherol to the CEP diluent strengthens the ability to maintain the quality of Brahman bull spermatozoa during storage at 4°C-5°C. It was similar to the SNI for AI until the seventh day of storing Brahman bull semen. The CEP media without α-tocopherol keeps the quality of bull spermatozoa from the rejected semen until the fifth day of storage. The increase in free radicals during the storage of spermatozoa at low temperatures may be due to the production of free radicals from dead or abnormal spermatozoa. The addition of antioxidants into the diluent media tends to minimize the production of free radicals [19].

Sperm stored at a low temperature can be damaged by free radicals. α-tocopherol is known as an antioxidant that reduces free radicals and can improve the quality of spermatozoa. We found that the addition of α-tocopherol into the CEP extender did not protect the spermatozoa of the Brahman bull at the beginning of storage. We observed no significant difference between the control and treatment groups. Furthermore, free radicals in the diluent can be overcome by antioxidants in spermatozoa cells during five days of storage. Therefore, the extent of damage to cells was low. α-tocopherol plays the role of an antioxidant and protects the cells or other compounds against free radicals by donating one hydrogen atom from the OH group to free radical compounds, resulting in tocopheroxyl radical compounds that are more stable and non-damaging. It can stop the propagation process when lipid peroxidation occurs in the membrane of spermatozoa [29]. The concentration of α-tocopherol can vary considering the percentage of lipids in the environment, affecting the solubility of vitamin E and the morphology of spermatozoa. Therefore, the type and composition of the diluent media and the type of animal will influence the dose of α-tocopherol [30].

There were significant differences in membrane integrity, motility, and viability on the sixth day of storage. These conditions were caused by free radicals in the diluent. Antioxidants were unable to reduce free radicals in the cell, especially in CEP diluents without adding α-tocopherol. The decrease in spermatozoa quality, such as motility, viability, and membrane integrity was faster in diluents without α-tocopherol compared to diluents containing α-tocopherol. Brahman bull spermatozoa stored in CEP diluents without α-tocopherol showed a low quality of sperm on the fifth day of storage. It was due to the presence of free radicals in semen. This finding is similar to buffalo sperm which has a higher quality with an extender containing α-tocopherol or vitamin C [31]. These results indicate that α-tocopherol provides the best protection for the plasma membrane of spermatozoa. α-tocopherol is known as an antioxidant that prevents lipid peroxidation during storage in bovine spermatozoa [32].

The integrity of spermatozoa DNA during storage was assessed based on the profile of the genes encoding spermatozoa motility (NAD1-1). Spermatozoa motility was a major parameter in determining spermatozoa quality at the center of AI. The motility parameter is determined by the ability of spermatozoa to fertil-

ize eggs. These results revealed no changes in genes during various treatments until day seven of storage. It indicated that the presence of free radicals during storage for up to seven days did not damage the cell and DNA structure. Gene sequences did not show any changes in either the control or treatment groups, before or after storage. The process of storing the liquid at 4°C-5°C was simpler than the freezing method and fewer free radicals existed in the liquid method than in the frozen technique. The freezing process can trigger damage to the integrity of the spermatozoa membrane, compared to storage at 4°C-5°C as happened in the freezing process of human spermatozoa. Freezing storage triggers damage in chromatin integrity by oxidative stress [33]. The storage of human spermatozoa at freezing temperatures damages DNA integrity [34]. DNA integrity did not damage after storage at 4°C-5°C, while DNA integrity was damaged after freezing [35].

Assessment of both sperm motility and sperm viability by light microscope was an important limitation of the present study. However, computer-aided sperm analysis technology (CASA) is more accurate for such assessments. Moreover, other concentrations of α-tocopherol or combinations of other antioxidants with α-tocopherol may be required to obtain better results on bull spermatozoa stored at 4°C-5°C. Therefore, further research is needed in this important field of research concerning spermatozoa preservations. It is necessary to study the effect of adding α-tocopherol to the CEP diluent in which each diluent has compositional characteristics, especially lipids that can affect α-tocopherol solubility. Consequently, it can influence the effectiveness of α-tocopherol in counteracting free radicals.

### *Conclusion*

The sperm motility, viability, and membrane integrity were higher in CEP with the supplementation of α-tocopherol. Our present study showed that lipid peroxidation was significantly different between the treatment and control groups. The CEP with α-tocopherol supplementation could maintain the spermatozoa quality during storage at 4°C-5°C.

## Materials & Methods

### *Preparation of Extender with α-tocopherol Addition*

The CEP extender in the present study was prepared according to Verbeckmoes et al. [18, 27] with antibiotic, egg concentration, and a different method (water jacket method) [20]. The following compounds were included in the CEP extender: BSA 2.0 gr/l, CaCl2(H2O)2 3.0 mmol/l, citrate acid mmol/l, fructose 55 mmol/l, KH2PO4 20.0 mmol/l, KCl 7.0 mmol/l, NaH2PO4 8.0 mmol/l, NaHCO3 11.9 mmol/l, NaCl 15 mmol/l, MgCl2(H2O)6 3.0 mmol/l, penicillin 1000 IU, streptomycin 1 gr, sorbitol 1.0 gr/l, and tris 133.7 mmol/l. Next, 2 mM of α-tocopherol (Sigma, USA) was added to the CEP extender while the control group was not supplemented with α-tocopherol.

### *Bull Semen Collection and Preparation*

Fresh semen of the bull was obtained from AI Center in Singosari, Malang, Indonesia by an artificial vagina. Bulls' semen was obtained twice a week to gain optimum semen quality. The fresh semen was observed to evaluate motility and viability before being diluted. The low quality of semen, according to the SNI, was continued for the process of dilution and freezing. If the quality was below the SNI, semen was rejected. According to the SNI, low-quality semen does not meet the requirements of the freezing process. For example, the motility of spermatozoa is less than 70% and the viability percentage is less than 75%, and it is called rejected semen. Fresh semen was diluted 25 times in a CEP extender with 2 mM α-tocopherol and without antioxidants as the control group. Spermatozoa were stored at refrigerator temperature (4°C-5°C) in darkness conditions at 25×106/ml concentration.

In the present study, we used fresh semen with low quality. We evaluated the level of spermatozoa degradation from fresh low-quality semen (rejected for the freezing process) during storage at low temperatures on CEP diluents with α-tocopherol as an antioxidant, compared to those without α-tocopherol.

### *Sperm Motility*

Spermatozoa motility was measured by observing semen using a light microscope to determine the percentage of progressive motility. Spermatozoa were taken by a stick glass on days 0 and 8 in the CEP-2 extender with and without egg yolk. Next, it was placed on an object glass and covered with cover glass. The observation was conducted under a light microscope with ×200 magnification. Evaluation of the motility-based method of Garner and Hafez [36] was performed by two people to determine sperm motility.

### *Sperm Viability*

Sperm viability was assessed using Eosin-Nigrosin staining to gain the permanent slides. Nigrosin provides a dark background to recognize viable cells. Non-viable sperms had red or dark pink heads and viable sperms had white or faintly-pink heads.

### *Membrane Integrity*

Membrane integrity was determined using the hypoosmotic swelling test. It was performed by incubating 100 µl semen in the control and treatment groups with 1 ml of 125 mOsm/l hypoosmotic (0.31 g sodium citrate and 0.565 g fructose in 50 ml H2O) at 37°C for 30 min. Afterwards, 0.2 ml of the mixture was spread using a coverslip on a warm slide after incubation. The observation was performed using 200 sperms under light microscopy at a magnification of ×400. Sperms with swollen or coiled tails were recorded [37].

### *Lipid Peroxidation*

Sperm oxidative levels were determined using the MDA assay-based TBA reaction. A total of 1 ml in each spermatozoa treatment (five technical replications) was incubated in 0.7 ml of tris buffer (100 mM, pH 7.4), 10 mM of iron sulfate, and 100 mM sodium ascorbate at 37°C for 60 min. This reaction was stopped by adding 1 ml of 10% trichloroacetic acid on ice for 15 min. Samples were immediately centrifuged at 7800 rpm for 15 min and the supernatant was removed. A total of 2 ml supernatant was add-

ed with 1 ml of 1% TBA into endogenous peroxidation. Next, the specimen was boiled for 10 min and kept in a cool condition. The absorbance levels were read by spectrometry at 530 nm [38, 39].

## *DNA Integrity*

**DNA extraction**

DNA extraction was conducted according to the method of Silva et al. [40]. Each semen treatment was divided into three aliquots. DNA was extracted with phenol-chloroform according to Hanson and Ballantyne [41]. A total of 100 µl of semen aliquots were centrifuged at 6000 rpm for 5 min. Pellet was resuspended using 1 ml Tris EDTA solution (1 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl, pH 8.0) and centrifuged at 6000 rpm for 5 min.

A volume of 500 µl of lysis buffer containing 25 mM EDTA, 0.5% sodium dodecyl sulfate, 100 mM NaCl, and 10 mM Tris-HCl with a pH of 8.0 was added to the pellet, followed by 22 µl of 0.1 M dithiothreitol and 25 µl proteinase K (QIAGEN GmbH). Then, the sample was incubated at 55°C for 3 h, with a vortex in each hour. A total of 500 µl phenol was added, balanced with Tris at pH 7.8, and followed by a vortex. Centrifugation was conducted at 10000 rpm for 3 min. The supernatant was transferred to another tube, along with 300 µl phenol and 300 µl chloroform, followed by vortex and centrifugation at 10000 rpm for 3 min. The supernatant was placed into a new tube and 700 µl chloroform was added. The mixture was centrifuged and gently vortexed again. The supernatant was placed into a new tube containing 95% ethanol. Samples were incubated at -20°C for 4 h. Each sample was centrifuged at 10000 rpm for 10 min, and the supernatant was removed. Then, the DNA pellet was dried and resuspended with 50 µl 1X TE buffer (100 mM Tris-HCl, pH 7.5, 0.25 M EDTA), and stored at -20°C until further analysis.

**DNA concentration and purity**

Genomic DNA concentration and purity were assessed by measuring optical density in a Genesys Spectrophotometer. DNA absorbance was read at 260 nm to determine DNA concentration. DNA purity was evaluated by determining the A260/280 ratio and comparing it with a reference value of 1.8 [42].

**DNA visualization on agarose gel**

The existence and quality of DNA genomes were analyzed with a 0.8% agarose gel. Five microliters of sperm pellets were stained with 0.3 µl of Blue-Green (LGC Biotecnologia, Cotia, SP, Brazil), and electrophoresis was performed on the agarose gel. The results were visualized under an ultraviolet transilluminator (Vilber Lourmat, Paris, France), and the image was digitalized (C7070; Olympus, Tokyo, Japan).

**DNA amplification**

Genomic DNA amplification with PCR used ND1 gene primers. The ND1 gene is 925 bp and the PE Biosystems ABI 3700 DNA Analyzer 96 capillary electrophoresis system could not accommodate the 925 bp sequence.

Table 5 presents the primers for segments 1 and 2 of the ND1 sequence.

The first segment of the ND1 sequence used the antisense primer (3'–5'), and the second segment used the sense primer (5'–3') to align the two segments. Primers were selected specifically for mtDNA to distinguish between mitochondrial and nucleic DNA.

## *Data Analysis*

Data including average motility, viability, and membrane integrity were analyzed by analysis of variance to determine whether the treatment caused an effect. Moreover, the Student's T-test (α = 5%) was utilized to assess the differences between treatments.

## *Fundings*

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## Authors' Contributions

ND: Conceptualization, Supervision, Formal analysis, Writing original draft; DH : Writing original draft, Review and Editing. Data Curation; WB : Methodolgy, Formal Analysis, Review and Editing; TS : Data Curation, Formal Analysis, Writing Original Draft; AA : Formal Analysis, Writing-Review and Editing, Methodology; SW : Methodology, Data Curation, Writing-Review and Editing.

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## Conflict of interest

The authors declared no conflict of interest for this research.

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**Table 5.**
Primers in the mtDNA sequencing procedure

| Segment | | Sense/antisense primer | Primer sequence |
|---|---|---|---|
| 1 | 457 bp (3131–3588) | BOVNADH1S (sense) | ATTCCCATCCTATTGGCC |
| | | BOVNADH1A(antisense) | GAGAGGGTAAAGGACCCACT |
| 2 | 488 bp (3567–4055) | BOVNADH1S (sense) | TAAGTGGGTCCTTTACCCTC |
| | | BOVNADH1A (antisense) | ATGTTTGTGGTGGGATGC |

species in liquid and frozen-thawed bufallo semen. Anim Reprod Sci. 2009;114(1-3):125-134. Doi: 10.1016/j.anireprosci.2008.10.002.

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# Effects of Oral Exposure to Titanium Dioxide Nanoparticles on the Liver, Small Intestine, and Kidney of Rats assessed by light microscopy and Transmission Electron Microscopy

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## ABSTRACT

The TiO2 NPs are widely used in many commercial products, nanomedicine, agriculture, personal care products, different industries, and pharmaceutical preparations with potential risks to human health and the environment. The present study investigated the effects of different doses of TiO2 NPs on the liver, small intestine, and kidney tissues in the rat. The TiO2 NPs were administrated daily through gavage at the doses of 10, 20, and 50 mg/kg BW for 2 months. A total of 32 male rats were divided into four groups. After 60 days, rats were euthanized with $CO_2$ gas (Code of Ethics: IR UM.REC.1400.327). Histopathological examination of the kidney, small intestine, and hepatic tissues treated with TiO2 NPs showed toxic changes compared to the control group. Histopathological examination revealed hemorrhage in the liver, swelling in the kidney glomerulus, as well as inflammation and damage to the mitochondria in enterocytes. Further evaluations are needed to understand the impact of different doses of NPs on human health.

## Keywords

*Small intestine, Kidney, Liver, Rat, TiO2 nanoparticles*

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## Abbreviations

*LM: light microscope*
*TEM: Transmission electron microscopy*
*TiO2 NPs: Titanium dioxide nanoparticles*



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## Introduction

Currently, the use of nanotechnology, including NPs, in various fields, such as medicine, cosmetics, energy, chemicals, and textile industries has increased significantly. The size of nanomaterials varies from 1 to 100 nm [2]. Some NPs, if they are based on certain metals, can interact with hydrogen peroxide present in cells, resulting in the production of hydroxyl radicals that can enter the nucleus and cause DNA damage. The TiO2 NPs are reported to be cytotoxic. Several reports have indicated that these NPs can induce oxidative stress and ROS leading to membrane and DNA damage. This structural damage causes apoptosis or genetic alteration in cells affecting the overall health of the organism [3]. NPs can enter through various routes such as the digestive or respiratory system and reach the blood or major organs. They can enter the body through various routes, such as the digestive or respiratory systems, and reach the blood or major organs. Therefore, it is necessary to understand the long-term effects of TiO2 NPs on various biological systems [4]. The TiO2 NPs are mostly used in a large panel of applications, such as manufacturing plastics, paints, cosmetics, sunscreens, and toothpaste, as an adjuvant in pharmaceutical pills, and as bleaching agents in the paper industry [3]. Oral absorption of TiO2 NPs depends on the particle type, size, surface charge, surface coating, protein binding, dose, and species. It may increase with smaller size, negative charge, and appropriate coatings. As for other engineering nanomaterials, the potential toxicological hazards of nano-sized TiO2 are related to intracellular bioaccessibility, the ability to react with macromolecules, and the generation of free radicals [4]. Toxicokinetic studies in rodents administered intravascular and oral NPs showed accumulation predominantly in the spleen, liver, intestine, and kidneys [5, 6]. Absorption of TiO2 can be different according to the exposure routes and there is little knowledge on how the kinetic relates to physicochemical characteristics, such as size [7]. Recent studies showed that TiO2 has very low oral bioavailability and slow tissue elimination which might result in the long run in tissue accumulation [8]. Moreover, a study performed on humans stated that TiO2 NPs are likely to agglomerate in gastric fluid, reducing the bioavailability in nanoform during oral exposure and there is no evidence of significant absorption regardless of particle size [9]. After short-term (5 days) oral exposure to TiO2 NPs (0, 1, and 2 mg/kg BW per day) in rats, deposition of TiO2 (aggregates of test nanomaterial) was shown in the spleen, selected as a putative indicator of TiO2 NP deposition in tissues [10, 11]. The gastrointestinal tract represents a route of entry for several NPs both directly through intentional ingestion or indirectly via NP dissolution from food contact materials or by the secondary ingestion of inhaled particles. In addition, the growing use of NPs may lead to increased environmental contamination and unintentional ingestion via water, food animals, or fish [12]. Due to the high use of NPs, we decided to study the histopathological effects of these particles on the intestinal, liver, and kidney tissues in the rat.

## Results

The present study explored the potential effects of TiO2 NPs on the jejunum, liver, and kidney following repeated oral administrations in rats, at doses relevant to human dietary intake. Detailed histological and morphometrical examinations of the jejunum, kidney, and liver were performed in the control and treated rats. No mortality occurred in any of the investigated groups of the current study. No effects on animal health and weight gain were observed during the treatments.

### *Kidney Histopathology*

Kidneys of all control rats demonstrated well-preserved and kept intact normal histological components of the glomeruli, renal tubules, and interstitial tissues of the cortex and medulla (Figure 1-A). Kidney histological analysis did not show any significant qualitative changes both in 10 and 20 mg/kg groups,

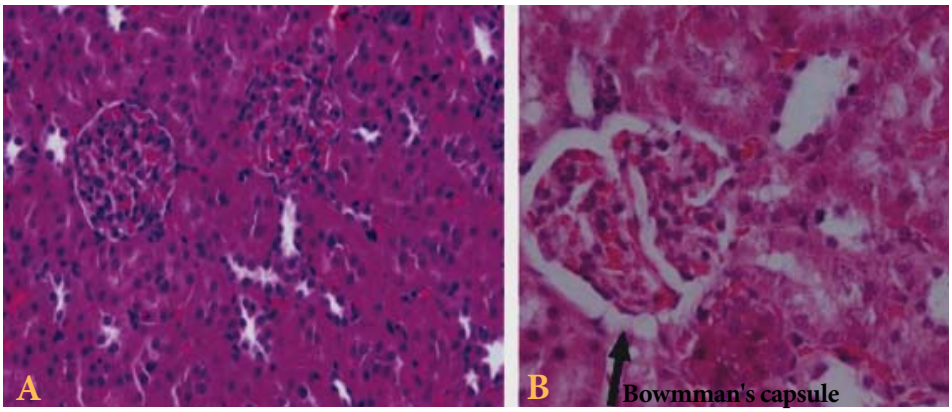


**Figure 1.**
A: Light micrograph of the kidney in control rats demonstrating normal histological architecture. H & E stain (×400). B: Light micrograph of the kidney in rats exposed to 50 mg/kg $TiO_2$ $NP_s$, demonstrating Bowman's capsule swelling and dilatation (arrow). H & E stain (×400).

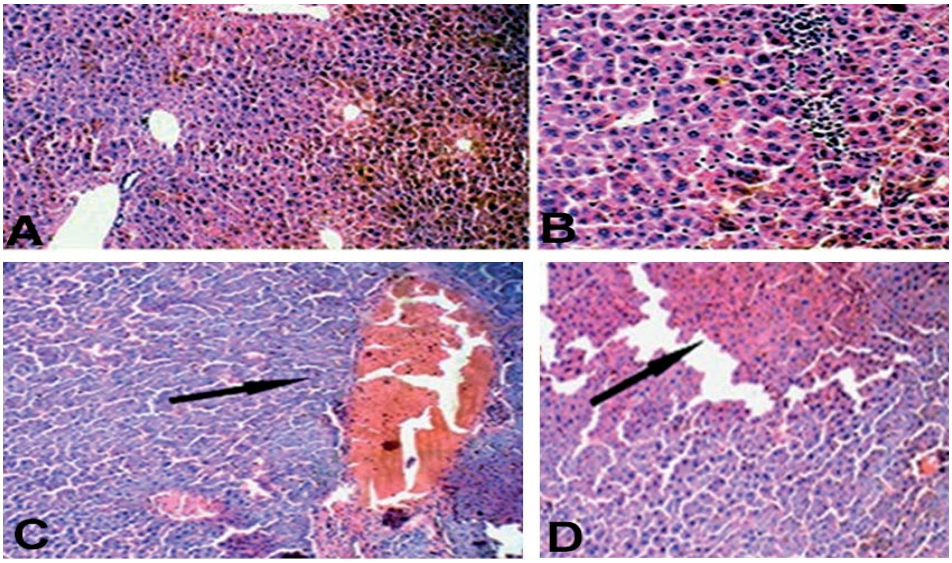


**Figure 2.**
A: Light micrograph of the liver in control rats demonstrating normal histological architecture. H & E stain (×400). B: Light micrograph of the liver of rats exposed to 10 mg/kg TiO2 NPs. H & E stain (×400). C: Light micrograph of the liver of rats exposed to 50 mg/kg TiO2 NPs. CV: Central Vein congestion (arrow). H & E stain (×400). D: Light micrograph of the liver of rats exposed to 50 mg/kg TiO2 NPs. The obstruction of central veins (arrow). H & E stain (×400).

However, in the group that received 50 mg/kg of NPs, Bowmman's capsule swelling and dilatation were observed. This study indicated the dissociation of junctions between the glomeruli and the renal tubule and might be associated with free radicals induced by TiO2 NPs exposure (Figure 1-B).

### *Liver Histopathology*

In the liver, no histopathological changes were observed in the untreated animals (Figures 2-A). After oral exposure, TiO2 NPs reached the liver via blood circulation. The NP aggregates were internalized in the Kupffer cells and probably into phagolysosomes localized in hepatic sinusoids as well as in the periphery of the portal tract. These different histological alterations occurred in animals exposed to the highest dose (50 mg/kg BW). In the histopathological study, no pathological lesion was observed and the liver had a normal appearance. Liver tissue was normal in the groups receiving 10 (Figures 2-B) and 20 mg/kg of NPs, but lesions, such as hyperemia and dilation in central veins, were observed in the group receiving 50 mg/kg (Figures 2-C and D).

### *Jejunum Histopathology*

Jejunum was completely normal in the control group (Figure 3-A) and in the groups that received 10 and 20 mg/kg of NPs. However, in the group that received 50 mg/kg of NPs, crypt structures were injured, the mucosa was eroded, and jejunum villi were loosened. Our results showed that in the TiO2 NP-treated groups, injured crypt structure, mucosal erosion, and the loosening of intestinal villi were present. The straight line shows the villus height (Figure 3-B). Absorptive cells were long and cylindrical. Basally located nuclei were in harmony with the shape of the cell. Microvilli were located on the luminal side of the cells (Figure 4-A).

### *Examining Histological Changes by Electron Microscopy*

After 60 days of oral exposure to TiO2, the ultrastructure of the absorptive cells showed significant changes. The most striking histopathological findings

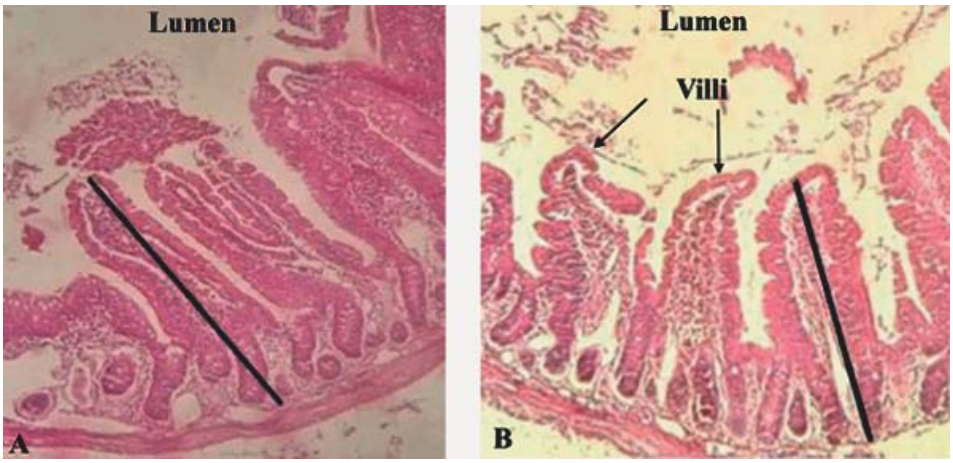


**Figure 3.**
A: Light micrograph of the jejunum of control rats demonstrating normal histological architecture. H & E stain (×400). B: Light micrograph of the intestine of rats exposed to 50 mg/kg TiO2 NPs. H & E stain (×400).

in the group of 50 mg/kg were distortion in microvilli and increased goblet cells and mast cells (Figure 5-A and B), mitochondrial elongation, along with excessive swelling of mitochondria and matrix dissolution (Figure 4-B).

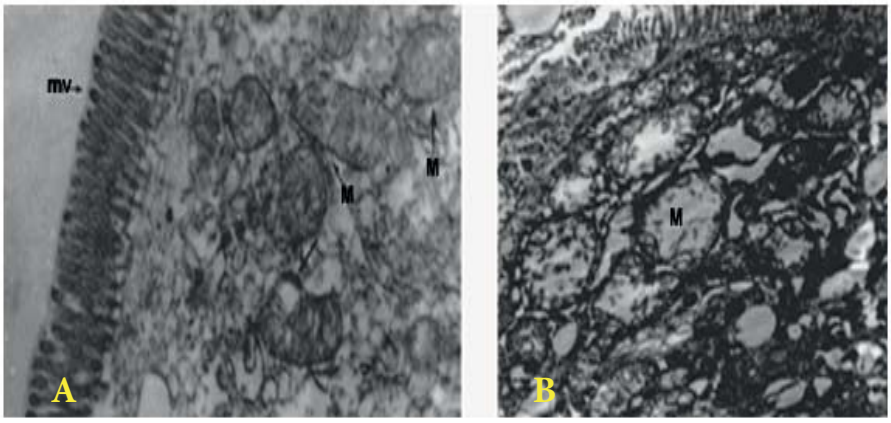


Figure 4.
A: Electron micrograph of the jejunum of control rats demonstrating normal histological architecture. Microvilli and apically located mitochondria in the control group. mitochondria (M) and microvilli (mv) (×20.000). B: Electron micrograph of the jejunum of rats exposed to 50 mg/kg TiO2 NPs. Mitochondrial cristae loss (M). Alterations in microvilli can be observed (×20.000).

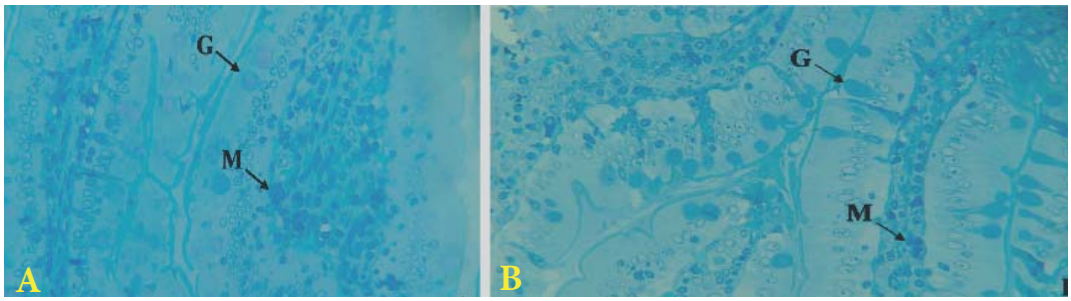


**Figure 5.**
A: Semi-thin micrograph of the jejunum of control rats demonstrating normal histological architecture. Goblet cells (G) and Mast cells (M) can be observed. B: Semi-thin micrograph of the jejunum of rats exposed to 50 mg/kg TiO2 NPs. An increase in Goblet cell (G) and Mast cell (M) numbers can be observed.

## Discussion

It is very challenging to draw firm conclusions on the toxicity of TiO2. Recent toxicity studies on TiO2 raised concerns for liver effects (fibrosis, steatosis, and edema) [13-18], and the potential promotion of intestinal tumors [16, 19-21] after ingestion. On the other hand, there are also toxicity studies showing no effect on the liver and intestine despite prolonged exposure and high doses [22, 23]. We included information from in vivo studies using advanced techniques to assess specific adverse effects in the liver, jejunum, and kidney. The toxic effects associated with TiO2 NPs in humans are mainly long-term effects resulting from chronic exposure by different routes (inhalation, ingestion, and transcutaneous passage from sun cream or internal production from titanium prostheses). The exposure of humans to TiO2 via different consumer products is estimated at 5 mg per person per day in occidental countries. Once incorporated into tissues, TiO2 nanoparticles are not eliminated and accumulate over time, which can lead to very high doses of several grams after several tens of years of exposure It is very difficult to recreate such chronic exposures in rodent models that have a short lifespan of no more than two years. Thus, most animal toxicity studies carried out on these NPs use different doses administered at a single time or over a relatively limited period. Daily, humans are exposed orally to TiO2 via food, food supplements, toothpaste (for young children), and medicines [24, 25]. Analysis of postmortem tissues indicated that these particles are taken up by the jejunum and are subsequently transported to secondary organs, such as the liver and kidney [26, 27]. Current legislation of the food additive E171 is based on the lack of effects in the chronic study by NCI (1979), investigating only traditional toxicological endpoints (NCI 1979). The small size of particles

enables them to enter and damage the organism by penetrating the physiological barriers traveling with circulatory systems [5]. A study found that oral exposure to the nano-forms of diverse particles was more toxic than micro-counterparts [28]. This study investigates the effect of TiO2 NPs on the kidney, jejunum and liver tissues.Nanoparticles can spread more in blood, kidney, liver and other organs [29]. This study agrees with Dhawan A et al. [30] who showed that oral exposure to TiO2 NPs causes apoptosis in the rat liver cells and induces severe oxidative stress. We concluded that TiO2 NPs can induce changes in the kidney, jejunum, and liver of rats. Liver damage may result from excess oral TiO2 NPs, which is in line with another study in 2012 [31]. The histo-toxicity of TiO2 NPs in high doses (50 mg/kg BW/day) was more than in low doses (10 mg/kg BW/day) in the kidney, jejunum, and liver tissues. Dietary exposure to TiO2 NPs caused liver toxicity [32]. Other investigations have demonstrated that pathogenic mechanisms initiated by some NPs were dominated by inflammation-driven effects, which occurred due to oxidative stress or DNA damage [5]. This explains the role of oxidative stress in cellular death by necrosis or degeneration in the rat kidney, jejunum, and liver treated with TiO2 NPs.

In the present study, animals were exposed to TiO2 NPs at different doses (10, 20, and 50 mg/kg BW). The rats were euthanized after 60 days to observe the chronic toxicological effects of TiO2. The range of doses used in our study was in accordance with other toxicological studies [33-35]. Morphological damage was induced by oral exposure to TiO2 NPs. In order to observe morphological changes, we opted for a high dose (50 mg/kg BW). The two lower doses (10 and 20 mg/kg BW) were used to detect histopathological changes.

### *Liver*

The liver is the major distribution site due to its high blood circulation and the phagocytosis of NPs by Kupffer cells. The other major target organs are the spleen, kidneys, and lungs [8, 33, 34]. The amount of TiO2 NPs in the mouse liver, spleen, lung, and kidneys reached high levels 14 days after intraperitoneal administration. Wang et al. in 2007 [7] also reported the accumulation of TiO2 NPs in the liver, kidneys, intestine, and lungs following oral administration. These alterations were correlated with oxidative stress localized in the same area of the liver. It may be the reason why hepatocytes present around the central vein are particularly sensitive to oxidative stress induced by TiO2. In conclusion, the present study highlighted the fact that TiO2 NPs caused detectable histological changes only in animals treated with high doses. In the liver, lesions affecting hepatocytes cells were related to oxidative stress.

### *Kidney*

In the kidneys, morphological alterations entailed the swelling of the renal glomeruli and therefore, probably more exposure to oxidative stress induced by TiO2 [10]. Bowman's capsule was the most affected part following exposure to the high doses of TiO2 NPs. These alterations were accompanied by changes in renal and hepatic function parameters that persisted chronically. In contrast, animals treated with lower doses showed no histological changes by light microscopy or significant variation in renal and hepatic function parameters. However, the very sensitive metabolomics approach allowed us to demonstrate a very early change in metabolism, even in animals exposed to the lowest doses of TiO2.

### *Jejunum*

At least four zones are distinguished in the intestinal tract, namely the duodenum, jejunum, ileum, and colon. The gut epithelium is composed of enterocytes and mucus-secreting cells (Goblet cells). The NPs are usually believed to be taken up by Goblet cells and M-cells, although this process is dependent on the particle size. In the current experiment, different doses of TiO2 were administered orally and electron microscopic assessments were carried out based on the control group to reveal the pathologic changes in the jejunum epithelium cells. Correlated with the exposure dose, the most significant changes were observed in mitochondria, goblet cells, and mast cells. Overall, the results of the present study showed that the gastrointestinal tract and enterocytes in particular may represent a target of TiO2 NPs toxicity following direct exposure. Ultrastructural changes in the intestine epithelial cells were observed using transmission electron microscopy, and severe structural damage was found in microvilli and mitochondria. It indicates that the possible site of the action of these TiO2 NPs is the cytoplasmic membrane and endomembrane system of the intestine epithelial cells. These findings provide a basis for the development of novel NPs active compounds with a novel mechanism of action. The intestinal compartment is highly chemically and physically complex. As a result, further studies are recommended to highlight the mechanism and mode of action for a reliable risk assessment of TiO2 NPs relevant to food safety.

## Materials & Methods

### *Nanoparticles Characterization*

The TiO2 NPs were purchased from Sigma-Aldrich, UK. The TiO2 NPs used in this study were titanium (IV) oxide and anatase with a purity of 99.7%. The TiO2 NPs were weighted and suspended in ultrapure water. In order to reduce the size of NP aggregates, NPs were sonicated in a probe sonicator for 3 runs of 30 min as detailed in the previous publications. Briefly, TiO2 NPs were characterized by a particle size analyzer and the titanium particle size was about 30 nm.

### *Animal and Treatment*

Thirty-two adult male rats at 2 months of age weighing approximately 220 g were used in this study. The rats were housed in cages under a regulated light and dark schedule on a 12 h day/night cycle and controlled ventilation, humidity, and temperature of 24°C ± 3°C and were fed with standard laboratory rodent pelleted feed and water ad libitum. After 5 days of acclimatization, the rats were distributed in four experimental groups of eight animals each (n = 8).

### *Treatment Groups*

Different doses of TiO2 were administered to experimental animals orally. The subjects were divided into four equal groups (n = 8). Doses were selected based on the available data on TiO2 NPs estimated human exposure and dispersibility of the test NPs in the selected medium, taking into account that the maximum volume administered by gavage to each rat cannot exceed 2 ml in aqueous solution. The TiO2 NPs were suspended in ultrapure water by sonication for 15 min and the dispersions were prepared daily. After 60 days, male rats were euthanized with CO2 gas (Code of Ethics: IR.UM.REC.1400.327). After dissecting the animals, tissue samples were taken from the liver, kidney, and intestine. The samples for LM were fixed in 10% buffered formalin. Next, the preparation of samples was performed by tissue processing. They were embedded in a paraffin dispenser, cut into 5-µm sections by semi-automatic microtome, and stained with Hematoxylin & Eosin. Briefly, the slides of tissues were examined by an image analysis system applied to an optical microscope. For electron microscopy, samples from jejunum were fixed in glutaraldehyde (3%) and osmium tetroxide. After the preparation of samples, they were blocked in Epon 812. Samples were cut by ultra-microtome and thin sections were double stained with saturated uranyl acetate (20 min) and lead citrate (10 min). Jeol JEM 100 CX-II electron microscope was used for examining the specimens.

Group I: Control animals received ultrapure water.
Group II: Received 10 mg/kg TiO2 NPs for 60 days.
Group III: Received 20 mg/kg TiO2 NPs for 60 days.
Group IV: Received 50 mg/kg TiO2 NPs for 60 days.

### *Ethics approval*

This study has the code of ethics (Code of Ethics for Study IR UM.REC.1400.327).

## Authors' Contributions

R J: Investigation; Writing-original draft; RJ , A R , A M J, H N:Conceptualization; Supervision; Writing-review & editing. All authors have been involved in writing the article, and accept responsibility for its content.

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## Conflict of interest

The authors declare that they have no conflict of interest

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# Phenotypic and Genotypic Characterization of Colistin Resistance in Escherichia coli Isolated from Bovine Mastitis

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## ABSTRACT

Mastitis is a global disease occurring in dairy cows, causing notable economic losses. Extensive use of antibiotics could allow the emergence of mobile antimicrobial resistance genes in mastitis-causing pathogens. This study aimed to investigate the prevalence and characterization of colistin resistance genes in *E. coli* recovered from bovine mastitic milk. A total of 74 *E. coli* isolates were investigated for antimicrobial resistance. The presence of *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* plasmid-mediated resistance genes, as the most crucial contributors to resistance to colistin, was examined by Multiplex PCR. Antimicrobial susceptibility patterns of all isolates to the seven most common antibiotics applied in dairy herds, including colistin, ceftriaxone, ampicillin, tetracycline, gentamicin, enrofloxacin, and trimethoprim-sulfamethoxazole were determined by the DD test. Among all samples, 70 isolates (94.6%) were resistant to colistin. In the MIC test, all isolates were also resistant to colistin, which was in agreement with the DD test. None of the *E. coli* isolates carried plasmid-mediated colistin resistance *mcr-1* to *5* genes in Multiplex PCR. Despite the important role of food-producing animals in the transfer of antibiotic resistance, mastitis-causing *E. coli* isolates were not the source of *mcr 1* to *5* genes in this study. The present research showed a high level of phenotypic resistance to colistin, while there was no agreement with their genotypic resistance. Consumption of polymyxins in dairy calves and the probable existence of other more effective resistance genes could be the reason for this high rate of phenotypic resistance.

**Keywords**

*Bovine mastitis, Colistin resistance, Escherichia coli, mcr gene, MDR*

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## Abbreviations

*E. coli: Escherichia coli*
*DD: Disk agar diffusion*
*MIC: Minimum inhibitory concentration*
*LPS: lipopolysaccharide*
*ESBL: Extended spectrum beta-lactamase*
*MDR: multi-drug resistant*
*CLSI: Clinical and Laboratory Standards Institute*



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## Introduction

Mastitis is considered a critical global condition in the dairy industry, causing notable economic losses due to various detriments, especially a significant reduction in milk production. The effects of mastitis on reproduction and product quality have been also documented [1]. Furthermore, clinical mastitis induces a vast range of symptoms from mild or moderate to severe with generalized signs, such as fever, anorexia, and pain, which are emergencies and should be instantly treated [2]. *E. coli* is one of the primary causative pathogens of mastitis, responsible for more than 80% of acute mastitis cases [3].

Colistin (also known as polymyxin E), which is a polypeptide with bactericidal activity against different species of Enterobacteriaceae, such as *E. coli*, targets the lipid A component of the LPS in the outer layer of Gram-negative bacteria [4, 5]. Oral formulations of colistin are usually used for intestinal disorders in calves. Intra-mammary colistin compounds also exist in the market to treat mastitis. Polymyxin resistance happens following changes in the lipid A moiety in the structure of LPS by either mutation in chromosomal genes or acquired resistance genes resulting in a more cationic LPS [3, 4]. Until 2016, chromosomal mechanisms leading to LPS modification, including augmented 4-amino-4-deoxy-L-arabinose (L-Ara4N), 2-aminoethanol, and phosphoethanolamine (PetN), or other approaches, such as capsule synthesis and efflux pump were considered the major reasons for colistin resistance attainment within Enterobacteriaceae [6]. The activation of PmrCAB and the two-constituent system PhoP/PhoQ due to mutation, inactivation, or mutation of the regulatory mgrB gene and consequent adverse feedback of the PhoP/PhoQ system causing lipid A modification in the LPS were recognized in animal E. coli isolates [3, 7]. The *mgrR* and *etk* encoding a tyrosine-kinase are other genes inducing colistin resistance in E. coli by altering the LPS charge [6].

Following the first record of the mcr-1 gene in 2016, many papers showed the presence of plasmid-mediated polymyxin resistance gene, which is coding mcr-1 phosphoethanolamine transferase on different plasmids in the isolates of animal, human, or environmental source in most countries [3, 4, 6, 8, 9]. Few retrospective studies have been conducted to separate mcr-1-positive isolates in the samples derived from chickens and calves in the 1980s and 2006, respectively. These studies revealed that the development of mcr-1-positive strains seemed to be a silent distribution of mcr genes during preceding decades rather than a current disaster [10, 11]. However, the growth of mcr-1 prevalence to 30% in 2014 from 5.2% in 2009 represented a striking raise in mcr-1 prevalence emphasized through the preceding years (11). The acquisition of the plasmid-mediated *mcr-1* gene currently has become the main reason for polymyxin resistance in *E. coli* as 98% of colistin-resistant *E. coli* can be described by the carriage of the plasmid-borne mcr-1 gene [12-14].

The announcement of a vast range of mcr-1-carrying plasmids in *E. coli* from various regions explains the potential of this gene to spread [13]. The mcr genes might spread quickly within important human pathogens due to the very high in vitro transfer of mcr-carrying plasmid among *E. coli* strains. The coexistence of *mcr-1* and genes encoding ESBLs and carbapenemases, namely CTX-M-55, CTX-M-15, and blaNDM, was observed in various sequences of *E. coli* isolates originated from several reservoirs [8, 11, 13,15]. Haenni et al. reported that 21% of recovered ESBL-producing *E. coli* samples had the mcr-1 gene with a higher frequency in veal calves [16].

The addition of transferrable *mcr-1* plasmid-mediated colistin resistance in carbapenem-resistant *E. coli* isolates, even in the absence of polymyxins' selective pressure, could be a global hazard of pan-drug resistant isolates development. However, the attainment of mcr-1 by *E. coli* could be a consequence of the substantial consumption of colistin in veterinary [3, 7, 14].

Co-occurrence of the *mcr-1* gene and resistance to various antibiotics, such as ampicillin, gentamicin, chloramphenicol, sulfonamides, trimethoprim, cephalosporins, and tetracyclines, has been reported [6, 11]. Extensive application of these antibiotics in veterinary medicine may have a role in distributing *mcr-1* and colistin resistance [11]. A 4- to 8-fold rise in the MICs of polymyxins may result from the presence of *mcr-1* in *E. coli* [4]. Considering the importance of colistin as the last-resort option for human infections caused by MDR bacteria and its broad consumption in veterinary medicine, the identification of mcr genes in food-producing animals is noteworthy in terms of public health concern that colistin resistance might be transmittable to humans [3, 4, 8].

Our study aimed to evaluate phenotypic resistance to colistin, the prevalence of plasmid-mediated colistin resistance genes (*mcr-1* to *5* genes), the relatedness of phenotypic and genotypic resistance, and also the agreement between two different phenotypic susceptibility tests in mastitis-causing *E. coli* isolates in a dairy farm.

## Results

### *Antimicrobial susceptibility tests*

A) Disk Diffusion: Based on the DD test, 70 *E. coli* isolates (94.6%) showed phenotypical resistance to colistin (zone diameter < 14 mm), which was the most prevalent resistance among all seven different antimicrobial agents. Enrofloxacin was the most effective agent compared to other antibiotics. Enrofloxacin inhibited bacterial growth in 62 isolates (83.8%) among all *E. coli* isolates. More details of phenotypical susceptibility to all seven antimicrobial agents are summarized in Table 1. Our study also revealed that 6 (8.1%) isolates were resistant to all seven antibiotics. Among all isolates (n = 74), 21 samples (28.4%) were known as MDR due to phenotypical resistance against at least three examined antibiotics other than colistin. Details of antibiotic susceptibility patterns to different antibacterial agents are summarized in Figure 1.

B) Minimum Inhibitory Concentration to colistin The MICs of isolates on cation-adjusted Mueller Hinton broth (Mueller Hinton broth 2) showed that all isolates were resistant to colistin (MIC > 8 µg/ml). Results also revealed that almost 42% of isolates (31 isolates) had MICs greater than 128 µg/ml.

### *Agreement between antimicrobial susceptibility tests*

MICs demonstrated that all isolates were phenotypically resistant to colistin, while DD results showed that 70 isolates (94.6%) were colistin-resistant (Table 2). There was a significant agreement between the two phenotypical susceptibility tests. The details are shown in Figure 2.

### *Occurrence of mcr genes in E. coli isolates*

In order to investigate the existence of mcr-1 to 5 resistance genes, Multiplex PCR was carried out, and the obtained results revealed that among all mastitis-causing E. coli isolates, none of the isolates carried *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* plasmid-mediated resistance genes.

## Discussion

The phenotypic colistin resistance of *E. coli* isolates in this research was 94.6% and 100% in DD and MIC, respectively. These two phenotypically colistin resistance tests had great concordance in our study. However, most studies showed that the DD test is unreliable and introduced standard broth microdilution as the golden standard for colistin resistance detection [17, 18]. There is a wide range of colistin resistance

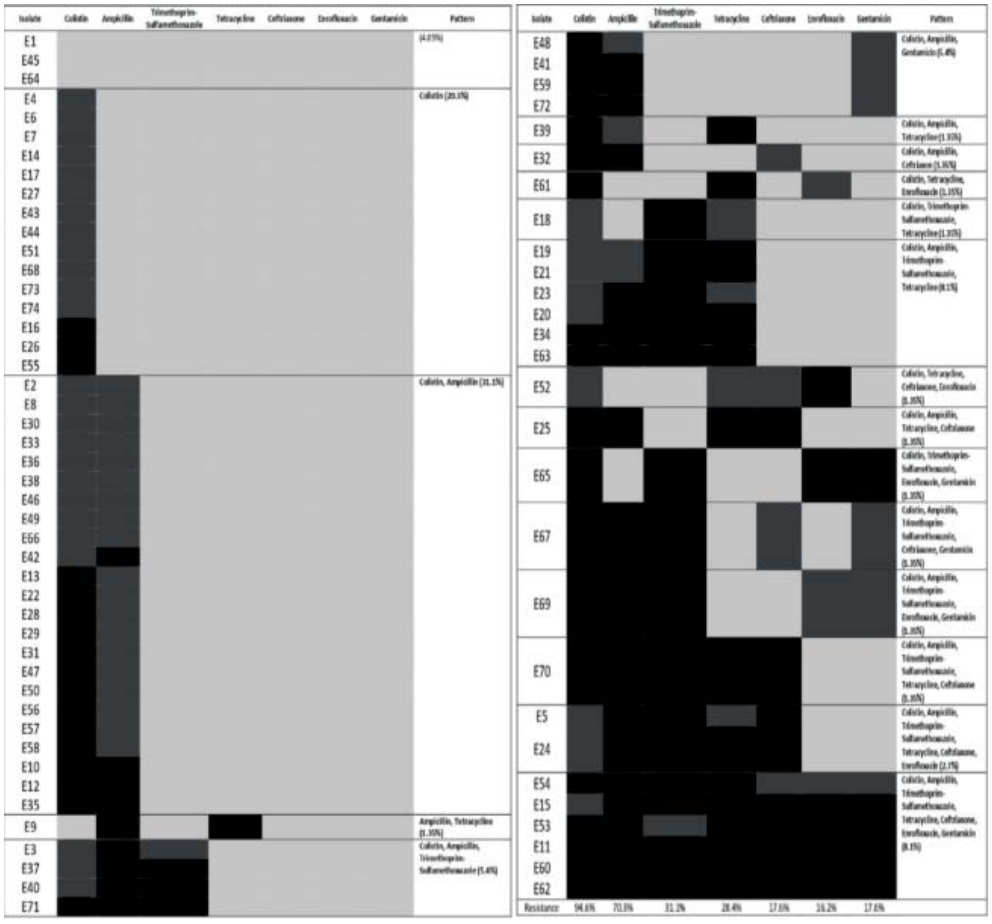

**Figure 1.**
Antibiotic Susceptibility Patterns of E.coli Clinical Isolates


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**Table 1.**
Comparison of phenotypic E. coli isolates susceptibility to 7 different antibiotics.

| Antibiotic Name | Susceptible (S) | Intermediate (I) | Resistant (R) |
|---|---|---|---|
| Tetracycline (TE) (30 µg) | 53 (71.6%) | 4 (5.4%) | 17 (23%) |
| Trimethoprim-Sulfamethoxazole (SXT) (1.25+23.75 µg) | 51 (68.9%) | 2 (2.7%) | 21 (28.4%) |
| Colistin (CL) (10 µg) | 4 (5.4%) | 34 (45.9%) | 36 (48.7%) |
| Gentamicin (GM) (10 µg) | 61 (82.4%) | 7 (9.5%) | 6 (8.1%) |
| Enrofloxacin (NFX) (5 µg) | 62 (83.8%) | 3 (4%) | 9 (12.2%) |
| Ampicillin (AM) (10 µg) | 22 (29.7%) | 23 (31.1%) | 29 (39.2%) |
| Ceftriaxone (CRO) (30 µg) | 61 (82.4%) | 4 (5.4%) | 9 (12.2%) |

**Table 2.**
Comparison of two phenotypically colistin resistance tests (Disk diffusion and MIC).

| | | | Disk diffusion results | | | Total |
|---|---|---|---|---|---|---|
| | | | susceptible | Intermediate | Resistant | |
| MIC (µg/ml) | 8 | Count | 1 | 0 | 0 | 1 |
| | | % of Total | 1.4% | .0% | .0% | 1.4% |
| | 16 | Count | 0 | 2 | 2 | 4 |
| | | % of Total | .0% | 2.7% | 2.7% | 5.4% |
| | 32 | Count | 1 | 11 | 6 | 18 |
| | | % of Total | 1.4% | 14.9% | 8.1% | 24.3% |
| | 68 | Count | 0 | 4 | 9 | 13 |
| | | % of Total | .0% | 5.4% | 12.2% | 17.6% |
| | 128 | Count | 0 | 5 | 2 | 7 |
| | | % of Total | .0% | 6.8% | 2.7% | 9.5% |
| | > 128 | Count | 2 | 12 | 17 | 31 |
| | | % of Total | 2.7% | 16.2% | 23.0% | 41.9% |
| **Total** | | **Count** | **4** | **34** | **36** | **74** |
| | | **% of Total** | **5.4%** | **45.9%** | **48.6%** | **100.0%** |

reported from different animals in various origins and locations, including five continents and forty countries [4, 8, 13, 15, 19]. High resistance to colistin (> 50%) was reported from piglets in Thailand. It was observed that 4.6% of avian E. coli isolates were colistin-resistant in Morocco. Resistance to colistin (3%-5%) in *E. coli* isolates from small animals was low in Sweden. Colistin resistance in bovine samples in Europe was 2% [8, 20, 21]. In Vietnam, 11% of the MDR *E. coli* strains derived from food animals were resistant to colistin [9]. The high rate of resistance to colistin in the current study compared to other investigations might result from orally administrated compounds of polymyxins to treat calves' intestinal disorders.

The screening of *E. coli* isolates from various animal species during 2000-2014 revealed that 1% of samples were classified as colistin-resistant cases. Generally, 0.4% of *E. coli* from several regions of the world were colistin-resistant cases (MIC ≥ 4 mg/L), whereas 32.2% of colistin-resistant isolates (overall prevalence: 0.1%) had *mcr-1* gene in 2014 and 2015 (13). A survey during 2010-2015 in Germany showed that 3.8% of *E. coli* isolates from various origins were resistant to colistin, while 79.8% of them had the *mcr-*

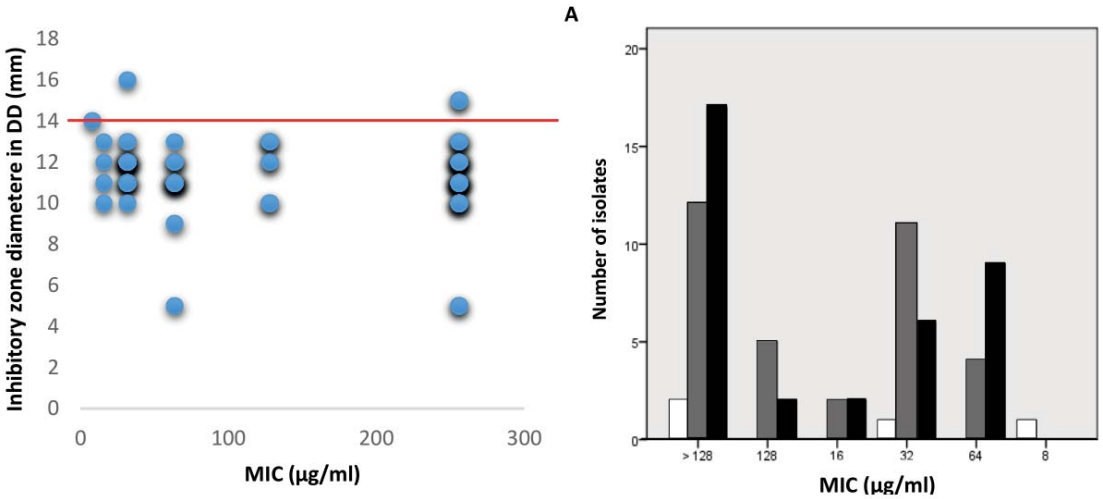


**Figure 2.**
Agreement between antimicrobial susceptibility tests

**Table 3.**
Primers and PCR conditions for molecular confirmation of isolates and mcr 1 to 5 detection.

| Genetic target | Primer | Primer sequence (5'-3') | Annealing temp. (⊠C) | Amplicon size (bp) |
|---|---|---|---|---|
| 23S rRNA | Eco223-F | ATCAACCGAGATTCCCCCAGT | 64 | 232 |
| | Eco455-R | TCACTATCGGTCAGTCAGGAG | | |
| *mcr*-1 gene | mcr1-fw | AGTCCGTTTGTTCTTGTGGC | 54 | 320 |
| | mcr1-rev | AGATCCTTGGTCTCGGCTTG | | |
| *mcr*-2 gene | mcr2-fw | CAAGTGTGTTGGTCGCAGTT | | 715 |
| | mcr2-rev | TCTAGCCCGACAAGCATACC | | |
| *mcr*-3 gene | mcr3-fw | AAATAAAAATTGTTCCGCTTATG | | 929 |
| | mcr3-rev | AATGGAGATCCCCGTTTTT | | |
| *mcr*-4 gene | mcr4-fw | TCACTTTCATCACTGCGTTG | | 1116 |
| | mcr4-rev | TTGGTCCATGACTACCAATG | | |
| *mcr*-5 gene | mcr4-fw | ATGCGGTTGTCTGCATTTATC | | 1644 |
| | mcr4-rev | TCATTGTGGTTGTCCTTTTCTG | | |

*1* gene. The *mcr-1* was also observed in 73.68% (14/19) of *E. coli* isolates collected from dairy cows in China.

The role of food-producing animals in spreading the mcr genes, even in healthy calves, was revealed in different countries [22]. In Belgium, the *mcr-1* to *mcr-5* genes were detected in healthy cattle, pigs, and poultry with the highest frequency of 77.5% (31 from 40 isolates) found for the *mcr-1* gene, 27 (67.5%) of which were carried out from cattle in 40 phenotypically colistin-resistant samples. In our study, although most isolates phenotypically were resistant to colistin, none of them had *mcr-1* to *5* genes [22, 23].

Amongst different sources, the prevalence of mcr genes in veal calves was low, and *mcr-1* was not detected in beef cattle, which is in agreement with our results [12]. The absence of mcr-positive isolates in our research was similar to other reports in human and animal specimens, such as bovine isolates, where these genes were not detected or the detection rate was low [8, 13, 24]. The mcr genes were reported the highest in the porcine and poultry collected isolates [25-27]. In contrast, the Islamic countries had no *mcr*

genes isolation or very low prevalence of mcr genes due to the lack of pig industry [28, 29]. In Saudi Arabia, mcr genes were not detected until the first report in 2016 [29]. In Iran, the first *mcr-1* gene detection from an animal source was in 2021 from a cow rectal swab, whereas no *mcr-2* to *mcr-6* genes were detected [30]. Ilbeigi et al. did not detect *mcr-1* and *mcr-2* genes in 36 bovine mastitis-causing and other 571 *E. coli* isolates of animal origin in Iran [28]. However, the presence of *mcr-1* in *E. coli* isolates recovered from cattle mastitis was reported in Egypt, Japan, and currently Greece [31-33]. Phenotypic resistance to colistin (with MIC ≥ 4 µg/mL) has been reported at 4% in bovine mastitis-causing Pseudomonas aeruginosa, and the *mcr-2* gene was also detected in two colistin-resistant isolates in Iran [34]. The prevalence of mcr-1-harboring *E. coli* isolated from bovine mastitic milk in China was 2% [24]. The first report of mastitis caused by ESBL-producing, *mcr-1*-harboring E. coli was recently in Greece, where the *mcr-1* gene was detected in 1.5% of isolates, while 22.25% of milk samples were phenotypically resistant to colistin [19, 31]. The high rate of phenotypic colistin resistance in our study could be related to its use in calves' digestive disorders treatment. Colistin constant consumption as well as other antibiotics, namely cephalosporins, cause the transfer of other resistance genes [7, 8, 11].

Although the detection of colistin-resistant E. coli isolates from ruminants or their products was not witnessed in some research, in 2014 the percentage of colistin resistance was estimated to be less than 2.5% for isolates from calves following cattle mastitis. The latter finding is contrary to the results of this study that only 5.4% of isolates were susceptible to colistin. In mastitis-causing Klebsiella pneumoniae strains, resistance to colistin was also reported by 1% in France [35].

Extensive use of cephalosporins, sulfonamides, and tetracyclines in veterinary medicine may also play a part in colistin resistance cases and even the distribution of mcr genes. Moreover, the co-occurrence of *mcr* genes with tetracyclines and sulfonamides resistance encoding genes was recorded [16]. Porcine *mcr-1*-harboring colistin-resistant *E. coli* isolates which simultaneously were resistant to ampicillin, gentamicin, sulfonamides, chloramphenicol, trimethoprim, tetracycline, or cefotaxime have also been reported [6]. Emerging colistin and carbapenems resistance in bovine mastitis-causing Pseudomonas aeruginosa was also recorded in Iran [34]. In our study, the higher percentage of MDR and pan-drug resistant isolates also confirmed resistance to colistin and other antibiotics. High rates of colistin resistance have been also noted among the strains of K. pneumoniae producing carbapenemase in Brazil and Italy but they lack mcr genes, which is in line with our results that phenotypic and genotypic resistance patterns were not compatible [36]. This is evidence of PCR limitation in which a negative result in PCR does not indicate susceptibility to colistin. PCR cannot exclude the chromosomal mechanisms of resistance, such as mutations, or even novel mcr genes not possessed in the test. Therefore, a negative PCR result for mcr genes would have insufficient predictive value for a colistin-susceptible phenotype [36].

### *Conclusion*

The current study indicated high phenotypic resistance to colistin in *E. coli* isolates from bovine mastitic milk and the significant concordance between two phenotypically colistin susceptibility tests MIC and DD. However, phenotypic and genotypic resistance patterns were not compatible. The high rate of colistin resistance may result from colistin use in dairy calves and its potential to induce resistance in mastitis-causing pathogens, such as *E. coli*. Despite the frequent usage of colistin in farm animals, the lack of mcr genes revealed that these genes were not widespread in veterinary and human clinical isolates in Iran, consistent with previous studies. Further investigations are also needed to understand the role of other colistin-resistance genes. The selection pressure of polymyxins in the dairy industry, even in calves, could provide a source of colistin resistance. Consequently, the possibility of other colistin-resistance genes' presence and their ability to spread to humans could be a global risk for public health. Hence, it should be noted that significant interruptions are required to lessen the spread of resistance to colistin in food animals.

## Materials & Methods

### *Sample collection*

The current retrospective cohort study was planned to investigate the prevalence of mcr-positive isolates among E. coli (n = 74) samples from mastitic cows in a dairy farm collected from October 2018 to February 2019. The severity status of all cases had been evaluated and recorded during sample collection. All isolates were collected based on National Mastitis Council guidelines. All milk samples were quickly transported on ice to the laboratory for microbiological culture.

### *Isolation and identification of E. coli*

Conventional bacteriological culture was performed based on the National Mastitis Council (1999). To this aim, 0.01 ml of milk was primarily overlaid on McConkey and Blood agar and incubated aerobically for 24 and 48 h at 37°C, respectively. A milk sample was described as positive if at least two colonies of any pathogen were observed on the plate. Plates with more than two different colony types were reported as contaminated samples. After morphological analysis of colonies, isolates were investigated by Gram staining. Supplementary metabolic and biochemical evaluations were performed as

needed applying particular microbiological analyses.
McConkey-positive samples were then subcultured on eosin methylene blue agar. In addition, sucrose and glucose fermentation, citrate, gas and $H_2S$ production, indole, and motility tests were performed to screen the samples for the existence of *E. coli*. Seventy-four *E. coli* confirmed isolates were finally selected to be included in the study.

### *Molecular confirmation of E. coli isolates*

To confirm the presence of *E. coli*, biochemically-positive samples were reanalyzed by PCR. For DNA extraction, 250 µl lysis buffer (0.2 M NaOH, 1% SDS, pH=8) and 250 µl Tris-EDTA buffer (100 mM Tris, 10 mM EDTA, pH=8) was first added to 200 µl of milk samples. Next, 550 µl phenol was added to the mixture. The supernatant was rinsed twice with phenol after 5 min centrifugation at 6000 rpm. Following the addition of 0.1 of 3.0 M sodium acetate (pH = 5.2), DNA was precipitated by ethanol and redissolved in distilled water after drying. Afterwards, 1 µg of extracted DNA was used to perform PCR. The primers were synthesized according to Riffon et al. (37). The details are given in Table 3.

### *Antimicrobial susceptibility testing*

**A) Disk Diffusion Test:** A total of 74 E. coli samples confirmed by bacteriological tests were selected to evaluate antibiotic susceptibility status to ceftriaxone (30 µg), colistin (10 µg), ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), enrofloxacin (5 µg), and trimethoprim-sulfamethoxazole (1.25+23.75 µg) disks by DD method. The diluted samples were equivalent to a 0.5 McFarland standard cultured on Mueller Hinton agar media. After overnight incubation at 37°C, the inhibitory zone was measured, and the susceptibility of samples was recorded by comparing to the standards of the CLSI and García-Meniño et al. study for colistin (17). However, the CLSI recommended the broth microdilution method as the gold standard for colistin susceptibility testing. We included the DD test in our with the cut-off value of ≤ 13 mm suggested by García-Meniño et al. to evaluate agreement with the gold standard method. Using a cut-off value of ≤ 13 mm, as inhibition zone diameter,have increased the sensitivity to 100% with a specificity of 98.7% (17). Isolates with antimicrobial resistance against at least three examined antibiotics other than colistin were considered MDR.

**B) Evaluation of minimum inhibitory concentrations of isolates:** To determine the MIC of isolates, the broth microdilution method was performed based on ISO standards for coliforms. Pure colistin sulfate powder (Sigma-Aldrich, Merck KGaA, Germany) was dissolved in distilled water and then kept at -80°C until the test, at the final dose of 1024 µg/ml. Cation-adjusted Mueller Hinton broth culture medium was poured into polyester pellets after preparation. For each of the tested isolates, eight serial concentrations of colistin from 1 to 128 µg/ml were added to the media. After overnight incubation, 50 µl of each sample equal to the standard concentration of 0.5 McFarland was added to each well and then incubated for 16-20 h at a temperature of 35°C ± 2°C. The MIC value was calculated based on the lowest concentration that completely inhibited bacterial growth.

### *Molecular detection of mcr-1 to 5 resistance genes*

The presence of *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* genes in all the isolates were analyzed by Multiplex PCR to evaluate the plasmid-mediated colistin resistance genes. All reactions were accomplished in a final volume of 25 µl. Multiplex PCR screened the existence of mcr-1 to 5 in isolates with the primers synthesized based on Rebelo et al. study (38). A volume of 1 µl of extracted DNA templates was added to 12.5 µl of master mix buffer solution, 10 ρmol of each 10 forward and reverse primers, and 9.5 µl of distilled water in a 0.5 ml microfuge tube. After applying a pre-PCR step at 94°C for 15 min, 25 cycles were run under the following condition: denaturation at 94°C for 30 sec, annealing at 58°C for 90 sec, and extension at 72°C for 60 sec. To finalize the reaction, the preparation was held at 72°C for 10 min following the last cycle. The details of the PCR protocol are summarized in Table 3. A 1.7% agarose gel stained with 0.5 mg of ethidium bromide/ml was used, and the agarose gel was finally visualized under UV light.

## Authors' Contributions

M.Z., S.H., H.M., M.A., and B.KH. conceived and planned the experiments. M.Z., S.H., and B.KH. carried out the experiments. M.Z. and K.I contributed to sample preparation. M.Z., M.A., and B.KH. contributed to the interpretation of the results. M.Z. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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CASE REPORT

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# Ectopic migration of Fasciola sp. in the lung of a water buffalo *(Bubalus bubalis)*

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## ABSTRACT

There are few reports of ectopic fasciolosis in the lungs of ruminants. Here, we report a rare case of ectopic pulmonary migration of *Fasciola sp*. in a water buffalo (*Bubalus bubalis*). In October 2021, during the routine veterinary inspection of the carcass and edible offal of a 3-year-old female buffalo in Tabriz industrial slaughterhouse, Iran, a severe liver infection with *Fasciola* parasites was observed. They were diagnosed as *Fasciola hepatica* based on size and gross morphology. One 3-cm fluke was recovered from the caudal lobe of the buffalo's right lung. Histopathologically, chronic active pneumonia, along with mild interstitial fibrosis, alveolar septa and pleura thickening, and necrosis associated with severe mixed inflammatory cell infiltration was observed in the affected lung. This case was observed for the first time in over 15 years of buffalo carcass inspection in the study area, indicating that pulmonary fasciolosis is rare in *Bubalus bubalis*.

**Keywords**

*Buffalo,* Fasciola, *Ectopic, Lung*

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## Abbreviations

No abbreviations



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## Introduction

Fasciolosis is a worldwide distributed zoonotic food- and waterborne infection. A wide range of domestic and wild animals as well as humans get infected mainly with *F. hepatica* and *F. gigantica* [1]. In addition, intermediate forms that occur after interspecific hybridization between these two species are present in geographical areas in which both species are endemic [2]. The economic impact of fasciolosis on livestock production, which is estimated to exceed 3 billion USD/year, results from sudden death due to acute infections, high condemnation of infected livers at abattoir inspection, costs of anthelmintic treatments, as well as a reduction in body condition score, milk yield, and live weight gains [2].

Mammalian hosts acquire the infection via ingesting food or water contaminated with metacercariae. Once in the small intestine, metacercariae excyst within an hour in the duodenum, releasing the juvenile stage, which penetrates the host's intestinal wall and appears in the abdominal cavity in approximately 2 hours. Most worms reach the liver within 6 days and migrate through the liver parenchyma for 5-6 weeks, preferentially feeding directly on the liver tissue. They eventually enter the bile duct where they mature sexually and initiate egg production [3]. Furthermore, it has been suggested that immature flukes may enter the bloodstream and be carried to various parts of the body or may reach the liver by traveling up in the bile duct. If the flukes fail to reach the hepatic biliary tree, they die in the abdominal cavity and other parts of the body [4].

Normally, the metacercariae of *Fasciola* cross the duodenal wall, migrate actively across the peritoneal cavity, and reach the bile ducts by penetrating Glisson's capsule and the liver parenchyma. However, some are lost on the way and occasionally grow in the peritoneal cavity or other ectopic foci. In humans, ectopic lesions are reported more frequently in the gastrointestinal tract and subcutaneous tissues. However, they are also found in the heart, blood vessels, pleural cavity, lung, abdominal wall, peritoneum, pancreas, spleen, stomach, appendix, cervical and inguinal lymph nodes, skeletal muscle, brain, eyes, and epididymis [3]. In ruminants, there have been few reports of ectopic fasciolosis in the lungs of cattle [5-8], sheep [9], and goats [10]. A relationship between foodborne trematodes and proximity to fresh water has already been demonstrated [11], which matches the habits of water buffaloes (*Bubalus bubalis*). To our knowledge, there are only two reports of erratic parasitism found in the lungs of water buffaloes in India, indicating the rarity of its occurrence [12, 13].

According to the latest official reports, nearly 224,000 water buffaloes live in 16 of 31 provinces of Iran. Almost 94% of buffaloes are reared in four provinces of Khoozestan, West Azerbaijan, Ardabil, and East Azerbaijan. They are raised traditionally and are used for meat and milk production. In 2020, approximately 27% (6549/24275) of slaughtered buffaloes were from East Azerbaijan province [14]. Herein, we report a case from a slaughtered buffalo in East Azerbaijan province, Iran.

## Case Presentation

In October 2021, during the routine veterinary inspection of the carcass and edible offal of a 3-year-old female buffalo in the Tabriz industrial slaughterhouse, a severe liver infection with *Fasciola* parasites was observed. Based on the size and gross morphology, the parasites were diagnosed as *F. hepatic*a. One mature *Fasciola* trematode ca. 3 cm was recovered from the caudal lobe of the buffalo's right lung (Figure 1). The lung tissue samples were collected for histopathological evaluation. The tissue was fixed in 10% buffered formalin, processed routinely, sectioned at 5 µm thickness, stained with hematoxylin and eosin (H&E), and finally, studied using a light microscope.

Microscopically, chronic active pneumonia in the affected lung was observed. in addition, mild interstitial fibrosis increased alveolar wall and pleura thickness, and necrosis associated with mixed inflammatory cell infiltration was found. Inflammatory cells included lymphocytes, macrophages, plasma cells, neutrophils, and eosinophils (Figure 2). Although no parasite sections were observed, the histopathological lesions were suggestive of a parasitic infection.

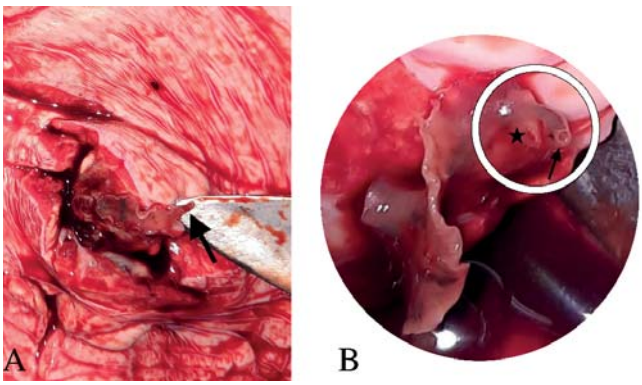


**Figure 1.**
Mature *Fasciola* helminth ca. 3 cm isolated from the caudal lobe of the right lung of water buffalo (*Bubalus bubalis)*; A) prominent anterior cone (arrow) and B) anterior end in focus. Note the oral sucker (arrow) and the acetabulum (asterisk).

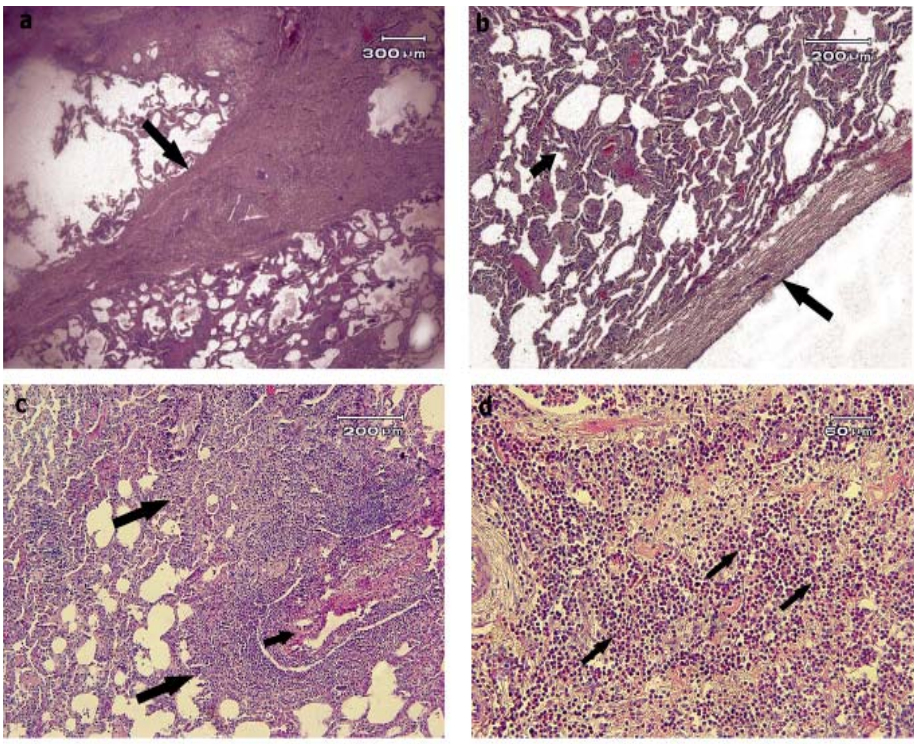


**Figure 2.**
Chronic active pneumonia was observed in the lung of the patient, H&E staining. Mild fibrosis (a: arrow), increased alveolar wall (b: small arrow) and pleura thicknesses (b: large arrow), and necrosis (c: small arrow) associated with mixed inflammatory cell infiltration (c: large arrows) were found. Inflammatory cells included lymphocytes, macrophages, plasma cells, neutrophils, and eosinophils (d: arrows).

## Discussion

Here, we report a rare case of ectopic migration of *Fasciola* sp. in the lung of a water buffalo (*Bubalus bubalis*). Ectopic manifestations usually appear shortly after infection. However, the route of migration toward ectopic sites is still unknown, and different hypotheses have been proposed. For instance, it has been suggested that migration occurs during the acute phase through blood vessels or soft tissues [8]. Further experimental studies using different infectious doses of metacercariae are needed to elucidate the mechanisms underlying ectopic migrations.

This case was observed for the first time during 15 years of buffalo carcass inspection in the study area, indicating that pulmonary fasciolosis is rare in *Bubalus bubalis.* There is only scarce information on the frequency of pulmonary fasciolosis in water buffaloes in the literature [12, 13]. In cattle, the predominant location of ectopic fasciolosis is the lung, and pulmonary fasciolosis has been observed in 2.2% of examined animals in Sudan, of which, one also showed pancreatic involvement [15]. Other researchers have reported erratic pulmonary fasciolosis in 3.7% and 4.1% of slaughtered cattle in Peru [5] and the US [8], respectively. The lower frequency of lung migrations in buffaloes needs further investigation.

Although the live fluke in this study did not reach the laboratory safely for morphometric and molecular examinations, based on the size and morphology (e.g., very prominent anterior cone), the worm could represent a young form of *F. hepatica* (Figure 1). It has been reported that ectopic flukes achieve maturity only rarely [3]. However, small lung nodules isolated from experimentally-infected calves were found either calcified or containing live specimens of *F. hepatica* [16]. In the latter study, live flukes recovered from the lungs were stunted in their development but sometimes contained mature eggs [16]. In contrast, in an abattoir-based study in Peru, flukes were pale pink to greyish-brown and sized 10.36 ± 3.44 mm long and 4.31 ± 0.97 mm wide, with no evidence of oviposition [5], suggesting that despite maturation in size, the flukes may not reach sexual maturity in the lungs.

Pathological lesions in this study were not specific, and no migratory track was present. In ectopic lesions, the common pathologic effects are due to the migratory tracks that cause tissue damage with inflammation and fibrosis. Flukes may then be calcified or contained in a granuloma [3]. In cattle and sheep, lung pathological alterations result from chronic irritation of the alveoli which are somehow related to the loss of their respiratory function followed by the thickening of the interalveolar septa or occlusion of the alveoli by foreign materials [6, 16]. Furthermore, secondary viral and bacterial infections may occur due to parasite migration into the lung parenchyma [6, 8] which were not observed in this case.

## Authors' Contributions

M.S. and Y.N. contributed to sample preparation. Y.N., M.S., D.M., M.K., A.R., and A.S. contributed to the interpretation of the results. A.S. took the lead in writing the manuscript.

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## Conflict of Interests

The authors declare that there is no conflict of interest.

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CASE REPORT

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# Uterine Horn Intussusception in a Chihuahua Bitch

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## ABSTRACT

Uterine horn intussusception has rarely been reported in bitches. However, the pathogenesis is not entirely clear and the incidence of uterine intussusception in bitches is unknown. This disorder occurs during the postpartum period and is a challenging clinical condition in terms of presentation, diagnosis, and management. In this report, a case of uterine horn intussusception is described in a four-year-old female Chihuahua bitch, which presented with abdominal pain, lethargy, anorexia, tremors, recumbency, and ongoing reddish vaginal discharge 20 days after whelping two pups. All the hematological and biochemical test results were within normal ranges except for a decreased calcium level. Ultrasonographic examination revealed the multilayered appearance of the uterine wall. The midline laparotomy confirmed the invagination of a proximal segment of the uterine horn into its distal segment near the uterine body. Ovariohysterectomy was performed as the recommended treatment. This case highlights the importance of assessing abdominal pain to avoid delay in diagnosis and management. Moreover, uterine intussusception should be considered as a differential diagnosis in bitches with the symptoms of abdominal pain during the postpartum period.

**Keywords**

*Bitch, Intussusception, Postpartum period, Uterine horn*

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## Abbreviations



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## Introduction

Different acquired disorders can affect the canine uterus during the postpartum period. Some of them include hemorrhage, retained placenta, sub-involution of placental sites, metritis, uterine prolapse, and rupture [1, 2]. Uterine intussusception is a rare acquired disorder in bitches [3]. Therefore, its incidence cannot be estimated exactly. The pathogenesis of uterine intussusception is not very clear. Physiological changes of the uterus during the postpartum period, such as uterus involution and hormonal changes or some pathological conditions, namely dystocia and retention of fetal membranes, may lead to uterine intussusception [3-7]. Diagnosis of this problem is a challenge because of the nonspecific clinical signs and symptoms. This report describes a case of uterine horn intussusception in a Chihuahua Bitch.

## Case Presentation

A four-year-old female Chihuahua was referred with a 24-hour history of lethargy, anorexia, tremors, and recumbency. The patient had a delivery 20 days before with an ongoing reddish vaginal discharge post-parturition. Her two previous pregnancies had been uncomplicated. She had no known trauma history. The bitch was nursing her puppies. On the physical examinations, although panting was initially observed, heart rate and rectal temperature were normal. In addition, some stiffness in her limbs and inability to stand was observed, and the palpation of the abdominal region was painful.

For hematological and biochemical assessment, blood samples were collected by venipuncture of the cephalic vein. The results of routine hematological and biochemical tests were within the normal range, except for a decreased calcium level (5.99 mg/dl, reference value: 9-10.8). In caudal abdominal ultrasonography, the multilayered appearance of the uterine wall (concentric rings) was obvious which represented the superimposed wall layers of intussusceptum and intussuscipiens. Furthermore, echogenic mesenteric fat was noticed in this view. The ultrasonographic findings were suggestive of uterine intussusception (Figure 1a). The surgeons advised a laparotomy, and the dog underwent surgery after her condition was stabilized by fluid therapy, perioperative pain management, oxygen therapy, and intravenous antibiotics. Confirmed surgical findings revealed the invagination of a proximal segment of the right uterine horn into its distal segment near the uterine body which confirmed uterine horn intussusception (Figure 1b). The manual reduction of intussusception was successfully performed by gentle pulling and the retrograde milking of the intussusceptum out of the intussuscipiens (Figure 2a). The intussuscipiens segment was distended without any significant abnormalities in the appearance of the affected uterine horn, and no adherence areas were observed (Figure 2b). An ovariohysterec-

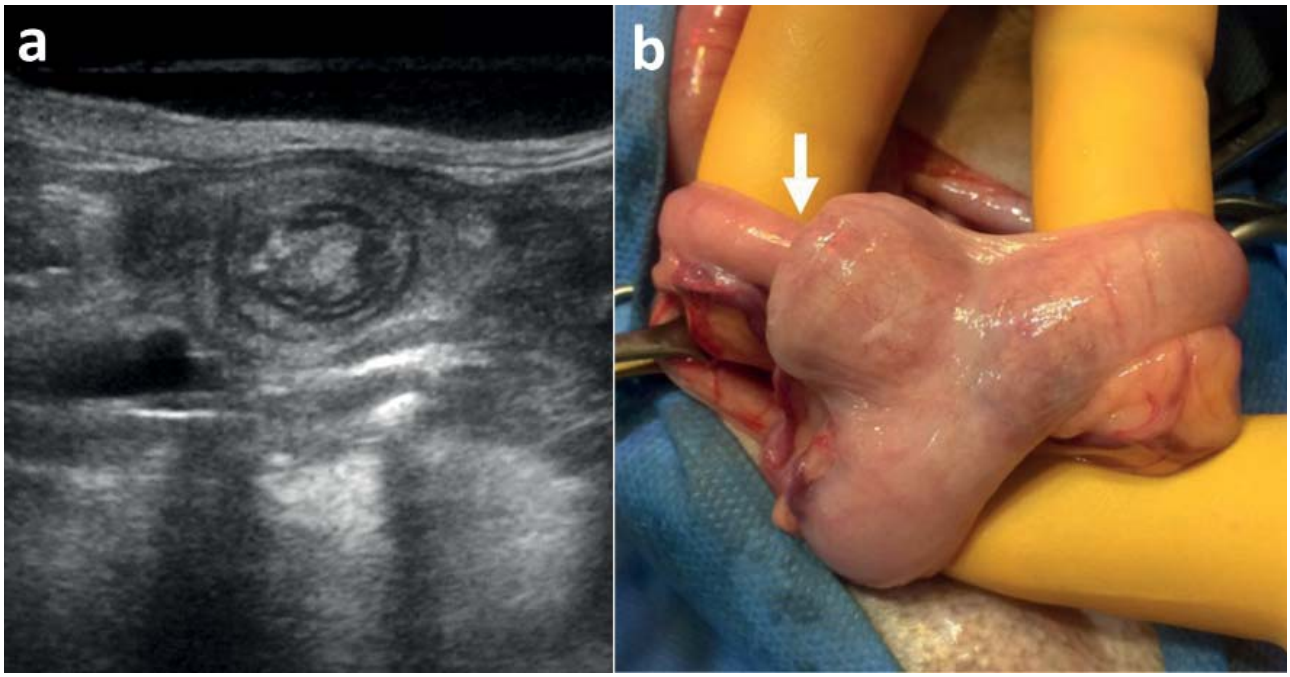


**Figure 1.**
a) Transverse ultrasonographic image of uterine intussusception, the inner uterine wall is identified within the outer uterine wall surrounded by echogenic mesenteric fat; b) Right uterine horn intussusception (arrow) near the uterine body

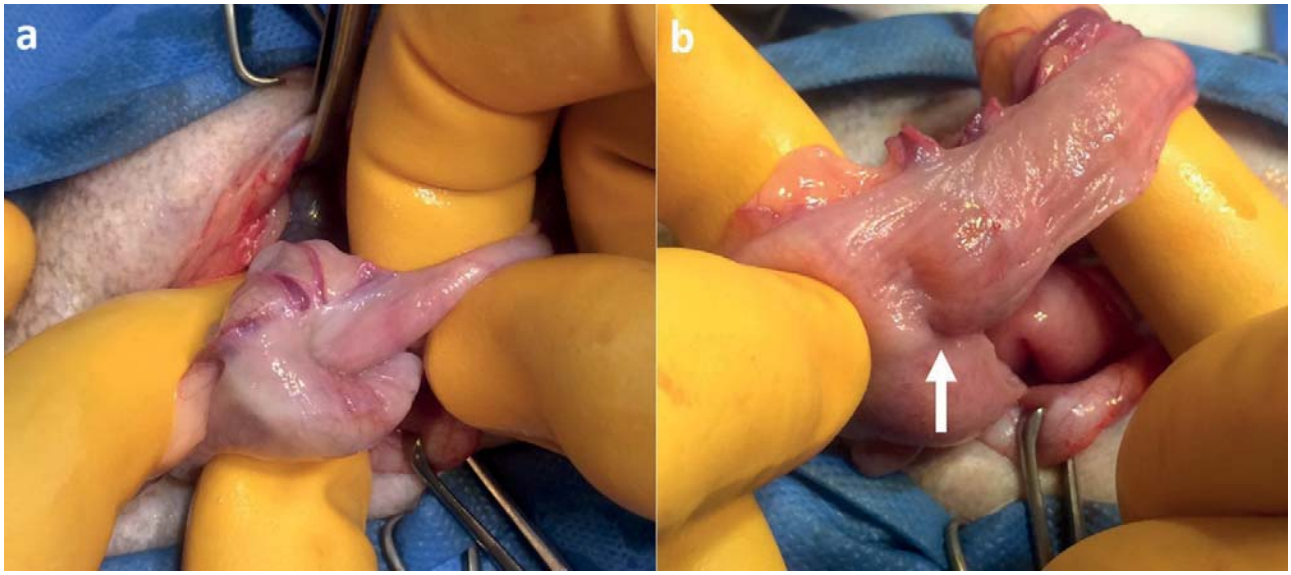


**Figure 2.**
a) Manual reduction of the invaginated uterine horn segment by a combination of milking and pulling; b) Uterine horn after the manual reduction of intussusception presenting a slight distention (arrow)

tomy was performed following obtaining the owner's consent.

## Discussion

Uterine intussusception is a rare condition in small animals, which has only been described in a few cases. To date, only less than ten case reports of bitches with this condition were published in the literature. Among these cases, only three were described in the English literature [3, 8, 9], while Brazilian veterinarians have reported three cases in Portuguese [3, 4, 8, 10, 11] and one case in Spanish [12]. The female dogs affected by uterine intussusception have been of various breeds, including Chow Chow [8], Golden Cocker [12], Yorkshire Terrier [10], crossbred [11], American Bully [3], Shih Tzu [4], German Shepherd [9], and Chihuahua. Uterine intussusception has also been previously reported in a nulliparous sheep [13] and a Thoroughbred broodmare [14].

The possible pathogenesis and complete clinical manifestations are not very clear due to the rarity of this condition. The tubular anatomical structure of the uterus and its ability for contraction and expansion may predispose it to intussusception [3]. In the cases of Chow Chow [8] and Yorkshire Terrier [10], the bitches were in the proestrus phase and imminent delivery, respectively. In other case reports [3, 4, 12], uterine intussusception was described in the postpartum period, as for a Chihuahua described in this paper. Therefore, it seems that uterine intussusception predominantly occurs during the postpartum period. This period is characterized by uterine involution which is completed when the uterus diameter returns to the original non-pregnant size. Furthermore, it was suggested that the normal involution process of the uterus is slow in the bitches and can last about 3 months [15]. During the postpartum period, physiological changes, involving the regeneration of the uterine tract [5], distention of uterine ligaments [3], and hormonal changes, including decreased or increased estrogen concentration [4], may be associated with uterine intussusception in this period. Moreover, uncoordinated peristalsis along the uterine tube may contribute to this condition [13, 16]. Uterine horn intussusception may be associated with prolonged dystocia because of the more atonic condition of the uterus [11]. Retained fetal membranes combined with prolonged straining and injudicious traction on a retained fetal membrane are other predisposing factors in this regard. The traction of a retained fetal membrane can invert the tip of the uterine horn, thereby leading to the progression of prolapse [3, 7]. However, in the reported case, parturition was normal.

In the Chihuahua Bitch presented in this paper, abnormally low levels of calcium were observed. Calcium is an essential element for the proper contractions of muscles, including the uterine muscles [17]. Low blood calcium is the leading cause of the poor contraction of the uterus, also known as uterine atony or inertia [2, 18]. Therefore, it seems that hypocalcemia may be implicated in the development of uterine intussusception via uterine inertia. Other hematological and biochemical values were normal in the case presented here. In a previous case of uterine intussusception, decreased hematocrit, the total number of red blood cells, and hemoglobin levels were found [3]. In contrast, in another case report, blood laboratory

analysis showed no changes in the parameters [9].

Uterine intussusception associated with non-specific symptoms makes it challenging to diagnose. The most commonly observed symptoms in previous cases [3, 4, 8-12] and the present case report are weakness, muscle tremors, dyspnea, abdominal pain, and vaginal discharge. Uterine prolapse was another concurrent clinical sign in a bitch [3]. Abdominal discomfort related to uterine intussusception may be caused by compromised circulation and pressure on nerve endings [7]. The uterine intussusception was detected in the right horn of the current case. This condition may develop either on the right horn or the left horn of the uterus. Uterine intussusception was previously reported three times in the right [3, 10, 11] and left [4, 8, 12] uterine horn.

In some previous case reports, the ultrasonography of the abdominal cavity was not performed or was not possible, and a definitive diagnosis was obtained by laparotomy [4, 10]. No signs of uterine intussusception were observed in one case of uterine intussusception in ultrasonography examination [3]. Therefore, it seems that pre-operative diagnosis is rather difficult in such cases. In contrast, the abnormal view of the uterus was visible in our case as the telescoping of concentric rings in ultrasonography was confirmed at surgery. A similar appearance in the abdominal cavity was reported in a case of uterine body intussusception in a bitch [9].

In large animals, such as horses, the manual reduction is often recommended for resolving uterine horn intussusception in affected mares. If the cases become nonresponsive, surgical intervention, including partial or total hysterectomy, is required [7, 19]. There is no defined non-surgical treatment for this condition in bitches. Ovariohysterectomy is the treatment of choice in small animals [20].

In conclusion, uterine intussusception must be considered as a differential diagnosis for abdominal pain in bitches during the postpartum period. This condition is quite rare, which limits the overall knowledge and understanding of this problem. Research on uterine intussusception epidemiology, pathophysiology, and diagnosis is required for effective management.

### *Ethical Approval*

This case reports details the management of a clinical case that was a part of clinical caseload. The owner understood procedure and agrees that results related to investigation or treatment and the high veterinary care has been performed with the consent of the animal owner.

## Authors' Contributions

All authors have been involved in writing the article, and accept responsibility for its content.

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## Conflict of Interests

The authors declare that there is no conflict of interest.

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# شناسایی مورفولوژی و ژنتیکی استروس اویس در گوسفندان در مناطق مختلف آب و هوایی ایران

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## چکیده

استروزیس یک نوع میاز بینی می‌باشد که در اثر هجوم لارو مگس استروس اویس به وجود می آید. ترشحات بینی و عطسه شایع ترین علائم بالینی در حیوانات آلوده است. لاروها به طور جدی سلامت میزبان را تحت تأثیر قرار می دهند و باعث کاهش تولید شیر، گوشت و پشم می شوند. این مطالعه بر روی گوسفند و بزهای سه منطقه آب و هوایی ایران (نواحی ساحلی دریای خزر، نواحی فلات کوهستانی و جلگه مسطح اطراف خلیج فارس) انجام شد. لاروها بر اساس کلیدهای تشخیصی معتبر شناسایی شدند. در مرحله بعد استخراج DNA از لاروهای جمع‌آوری شده انجام گردید. تکثیر ژن میتوکندریایی COI به منظور شناسایی تفاوت‌های ژنتیکی و آنالیز فیلوژنی از DNA ی استخراج شده لاروها انجام شد همچنین مطالعات هاپلوتیپ و درخت فیلوژنی در توالی‌های ژن ذکر شده انجام گردید. واکنش PCR که تکثیر قطعه ۶۱۰ جفت باز ژن میتوکندریایی COI را هدف قرار داد، گونه مورد نظر را تایید کرد. توالی‌های نوکلئوتیدی COI بدست آمده از این مطالعه در بانک ژن جهانی ثبت و سپس شماره‌های دسترسی به آن‌ها اختصاص داده شد. نتایج توالی‌یابی جدایه‌های استروس اویس از مغز گوسفندان در این مطالعه نشان داد که تفاوتی در ژن COI در بین جدایه‌ها وجود ندارد و شباهت زیادی با توالی‌های جدایه‌های استروس اویس از عراق، بوسنی، هرزگوین و کرواسی دارد. مجموعه اطلاعات مولکولی در مورد گونه‌های استروس اویس برای اولین بار در این مطالعه ارائه گردید که یک پایگاه داده مهم برای ارزیابی روابط فیلوژنی و شناسایی مولکولی این انگل‌ها ارائه می‌دهد.

## واژگان کلیدی

فیلوژنی و توالی ،PCR ، استروس اویس، گوسفند، سیتوکروم اکسیداز ۱

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# توزیع مقاومت ضد میکروبی و برخی از ژن های شایع بتالاکتاماز وسیع الطیف در فیلوگروه های مختلف جدایه های اشرشیا کلی تولید کننده سم شیگا (STEC) با منشأ نشخوارکنندگان

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## چکیده

داده های کمی پیرامون شیوع ژن های بتالاکتاماز وسیع الطیف (ESBL) در جدایه های اشرشیا کلی تولید کننده سم شیگا (STEC) با منشأ نشخوارکنندگان وجود دارد. این مطالعه، شیوع مولکولی ژن های رمز کننده ی ESBL (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ و bla$_{OXA}$) و مقاومت ضد میکروبی ۵۸ جدایه STEC بدست آمده از گاو (۳۲)، گوسفند و بز (۲۶) را تعیین می کند. در مطالعه حاضر، ژن های ESBL با روش مولکولی شناسایی شدند؛ در حالی که، مقاومت ضدمیکروبی علیه شش آنتی بیوتیک (آموکسی سیلین-کلاوولانیک اسید، تتراسایکلین، نئومایسین، فلورفنیکول، انروفلوکساسین و سولفامتوکسازول-تری متوپریم) با استفاده از روش انتشار دیسک آزموده شد. همچنین، گروه های فیلوژنتیکی با استفاده از PCR مشخص گشت. جدایه ها در پنج فیلوگروه (A، B1، C، D و E) قرار گرفتند و B۱ شایع ترین گروه (۴۳؛ ٪ ۷۴/۱) شناخته شد. همچنین، ارتباط معنادار بین فیلوگروه D و نشخوارکنندگان کوچک (گوسفند و بز؛ $p$ = ۰/۰۱۴ ) آشکار شد. به علاوه، بیش ترین نرخ مقاومت ضد میکروبی مرتبط با تتراسایکلین (٪ ۲۵/۹) و نئومایسین (٪ ۲۲/۴) بود. همچنین، جدایه های مقاوم به تتراسایکلین ($p$ = ۰/۰۰۱ )، تری-متوپریم-سولفامتوکسازول ($p$ = ۰/۰۱۳) و نئومایسین ($p$ = ۰/۰۰۰) به طور معناداری در میان سویه های بدست آمده از گاو شایع-تر بودند. علاوه بر این، سویه های دارای مقاومت چندگانه نیز در میان سویه های گاوی به صورت معناداری ($p$ = ۰/۰۰۱ ) حضور داشتند. در مطالعه حاضر، شیوع STEC های دارای ۱۲/۰۶ ٪ ESBL، بود. ژن های *bla*$_{CTX-M}$ و *bla*$_{TEM}$ به صورت جدا و در ترکیب با یکدیگر در سویه های گاوی شناسایی شدند. با این حال، تنها یک سویه ی مرتبط با نشخوارکنندگان کوچک، دارای *bla*$_{TEM}$ بود. در نهایت این طور به نظر می رسد که جدایه های گاوی منابع قابل توجهی از مقاومت های ضد میکروبی هستند که می تواند خطری برای واحدهای دامپزشکی، بهداشت عمومی و به خصوص بهداشت مواد غذایی باشد.

## واژگان کلیدی

اشرشیا کلی،STEC, (AMR)مقاومت ضد میکروبی *bla*$_{TEM}$، *bla*$_{CTX-M}$، ESBL، فیلوگروه

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# ارزیابی رادیوگرافی و بافت شناسی اثرات ملوکسیکام و فلونیکسین مگلومین بر روند ترمیم نقیصه‌ی تجربی استخوان زند زبرین خرگوش

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## چکیده

هدف از این مطالعه بررسی اثرات داروهای ملوکسیکام و فلونیکسین مگلومین بر روند ترمیم طبیعی استخوان زند زبرین خرگوش متعاقب نقیصه‌ی تجربی بود. در این مطالعه نود سر خرگوش نر سفید نژاد نیوزیلندی با میانگین سنی ۱۲-۱۰ ماه و محدوده وزنی ۲/۶-۱/۵ کیلوگرم به صورت تصادفی در سه گروه قرار داده شدند. متعاقب بی‌هوشی هریک از خرگوش‌ها با کوکتل زایلازین و کتامین، نقیصه‌ای به قطر و عمق ۳ میلی‌متر در سطح داخلی استخوان رادیوس اندام حرکتی قدامی چپ ایجاد گردید. در گروه اول، ملوکسیکام و در گروه دوم فلونیکسین مگلومین و در گروه کنترل مثبت، سرم فیزیولوژی در یک دوره درمانی ۱۰ روزه به شکل زیر جلدی تجویز شد. ارزیابی ترمیم نقیصه‌ها با اخذ رادیوگراف‌ها و تهیه مقاطع هیستوپاتولوژی انجام گرفت. ارزیابی تصاویر رادیوگرافی نشان داد که در روزهای صفر و هفت در هر سه گروه اثری از تشکیل کالوس در موضع نقیصه‌ی استخوانی مشاهده نشد. آغاز روند پر شدن نقیصه در گروه سالین و ملوکسیکام از روز ۱۴ پس از جراحی و در گروه فلونیکسین مگلومین از روز ۲۱ بود. در روزهای ۱۴ و ۲۱ گروه ملوکسیکام نسبت به گروه فلونیکسین مگلومین از نظر تشکیل کالوس و پرشدگی نقیصه، میانگین امتیازی بیشتری کسب نمود اما این میانگین امتیازی در گروه فلونیکسین مگلومین در روز ۲۸ بیش از گروه ملوکسیکام بود و بعد از آن این نسبت تا انتهای مطالعه در گروه ملوکسیکام بیشتر بود. این مطالعه نشان داد که ملوکسیکام نسبت به فلونیکسین تأثیر کمتری در تأخیر روند ترمیم استخوان دارد.

## واژگان کلیدی

رادیوگرافی، هیستوپاتولوژی، ملوکسیکام، فلونیکسین مگلومین، خرگوش

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# اثرات مواجهه خوراکی با نانوذرات دی اکسید تیتانیوم بر کبد، روده کوچک و کلیه در موش صحرایی توسط میکروسکوپ نوری و الکترونی

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## چکیده

نانو ذرات دی اکسید تیتانیوم به طور گسترده در بسیاری از محصولات تجاری، نانو پزشکی، کشاورزی، صنایع مختلف و فرآورده های دارویی با خطرات بالقوه برای سلامت انسان و محیط زیست استفاده می شود. مطالعه حاضر اثرات غلظت های مختلف تیتانیوم را بررسی کرد. نانوذرات دی اکسید تیتانیوم بر روی بافت کبد، روده کوچک و کلیه در موش صحرایی به عنوان دوز خوراکی روزانه (۱۰، ۲۰، ۵۰ میلی گرم بر کیلوگرم وزن بدن) به صورت گاواژ به مدت ۲ ماه تجویز شد. ۳۲ موش صحرایی نر به چهار گروه تقسیم شدند. پس از ۶۰ روز، موش‌ها با گاز دی اکسید کربن کشته شدند (Code of Ethics for Study IR UM.REC.1400.327). بررسی هیستوپاتولوژیکی بافت‌های کلیه، روده کوچک و کبد تیمار شده با نانو ذرات دی اکسید تیتانیوم تغییرات سمیت را در مقایسه با گروه کنترل نشان داد. نتایج هیستوپاتولوژیک خون ریزی در کبد، تورم گلومرول در کلیه، التهاب و آسیب به میتوکندری در سلول های انتروسیتی را نشان داد. برای دانستن تأثیر دوزهای مختلف نانو ذرات بر سلامت انسان به مطالعات بیشتری نیاز است.

## واژگان کلیدی

روده کوچک، کلیه، کبد، موش صحرایی، نانو ذرات دی اکسید تیتانی

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# خصوصیات فنوتیپی و ژنوتیپی مقاومت به کلستین در اشرشیاکلای جدا شده از ورم پستان گاوی

**محمدهادی زارعی[1]، سعید حسین زاده [2]، هادی محب علیان[3] ، محمد عزیززاده [1] ، کیانا ایراندوستی[1]، بابک خرمیان*[1]**

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## چکیده

ورم پستان یک بیماری جهانی قابل ملاحظه است که در گاوهای شیری باعث خسارات اقتصادی چشمگیری می شود. استفاده گسترده از آنتی بیوتیک ها می تواند باعث ظهور و گسترش ژن های مقاومت آنتی بیوتیکی در عوامل بیماری زای ایجاد کننده ورم پستان شود. این مطالعه با هدف بررسی شیوع و خصوصیات ژن های مقاومت به کلستین در نمونه های *E. coli* جدا شده از شیر گاوهای درگیر ورم پستان انجام شد. در این مطالعه، مجموعا ۷۴ جدایه *E. coli* جهت مقاومت آنتی بیوتیکی مورد بررسی قرار گرفت. حضور ژن های *mcr 1* تا 5 نیز، به عنوان مهمترین عامل مقاومت در برابر کلستین، با روش MULTIPLEX-PCR بررسی شد. الگوهای حساسیت آنتی بیوتیکی همه جدایه ها به هفت آنتی بیوتیک متداول در گله های گاو شیری، از جمله کلستین، سفتریاکسون، آمپی سیلین، تتراسایکلین، جنتامایسین، انروفلوکساسین و تری متوپریم-سولفامتوکسازول با تست دیسک آنتی بیوتیکی (DD) تعیین شد. در میان تمام جدایه ها، هفتاد نمونه (۶/۹۴ درصد) به کلستین مقاوم بودند. در آزمون تعیین حداقل غلظت مهاری، همه نمونه ها به کلستین مقاوم بودند که با نتایج تست دیسک (DD) تطابق داشت. در آزمون Multiplex-PCR در هیچکدام از جدایه ها ژن های مقاومت *mcr 1* تا 5 شناسایی نشد. علیرغم نقش مهم حیوانات تولیدکننده غذا در انتقال مقاومت آنتی بیوتیکی، عوامل اشرشیاکلای مسبب ورم پستان در گاو شیری منبع ژن های مقاومت به کلستین *mcr 1* تا 5 در این مطالعه نبودند. این مطالعه سطح بالایی از مقاومت فنوتیپی به کلستین را نشان داد؛ در حالی که، همخوانی با مقاومت ژنوتیپی آنها وجود نداشت. مصرف پلی میکسین در گوساله های شیری و وجود احتمالی ژن های مقاومت دیگر می تواند دلیل این میزان بالای مقاومت فنوتیپی باشد.

## واژگان کلیدی

ورم پستان گاوی، مقاومت به کلستین، ژن *mcr* ،اشرشیاکلای , MDR

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# درهم روی شاخ رحم در یک قلاده سگ ماده شی‌هواهوا

**سمانه قاسمی*، محمدرضا امامی، علی میرشاهی، پوریا خان‌زاده، سینا یل بیرانوند، محمدمهدی زارع‌زاده**

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## چکیده

درهم‌روی شاخ رحم به‌ندرت در سگ‌های ماده گزارش شده است که پاتوژنز آن به‌طور کامل آشکار نیست. به‌هر حال بروز درهم‌روی شاخ رحم در سگ‌های ماده ناشناخته است. این اختلال در طی دوره پس از زایمان رخ می‌دهد و از نظر تظاهر، تشخیص، و مدیریت وضعیت بالینی چالش‌برانگیز است. در این گزارش، یک مورد از درهم‌روی شاخ رحم در یک سگ ماده شی‌هواهوا چهارساله که با درد شکمی، بی‌حالی، بی‌اشتهایی، لرزش، زمین‌گیریف و ترشحات واژنی قرمزرنگ مداوم بیست روز پس از زایمان دو توله مراجعه کرده بود، شرح داده می‌شود. تمامی نتایج هماتولوژی و بیوشیمی به جز کاهش سطح کلسیم در محدوده نرمال بودند. معاینه سونوگرافی، نمای چندلایه دیواره رحم را نشان داد. لاپارتومی خط وسط فرورفتگی قطعه بالایی را در داخل قطعه پایینی نزدیک بدنه رحم تایید کرد. اواریوهیسترکتومی به‌عنوان درمان توصیه شده انجام شد. این مورد اهمیت ارزیابی درد شکمی را به‌منظور تاخیر در تشخیص و مدیریت برجسته می‌کند. درهم‌روی رحم باید به‌عنوان تشخیص تفریقی در سگ‌های ماده در دوره پس از زایمان باید مورد توجه قرار بگیرد.

## واژگان کلیدی

سگ ماده، درهم‌روی، دوره پس از زایمان، شاخ رحم

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# Author Index



## Author index

# Guide for authors

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### *Materials and methods*

Materials and methods should be described in sufficient details to allow other researchers to reproduce the results. Specify any statistical computer programs used .The methods of data collection and use of statistical analysis will be checked by the referees and if necessary, a statistician. Drugs and therapeutic agents, reagents, softwares and equipments should be given in the format: name (trade name, manufacturer name, city, country), e.g. Statview 5 (SAS Institute, Inc., Cary, NC, USA).

**Animals:** All animal experiments should comply with the ARRIVE (https://arriveguidelines.org/) guidelines and the authors should clearly indicate in the manuscript the ethical code of the study.

**Gene names:** The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

Mouse beta actin gene: *Actb*
Bovine beta actin gene: *ACTB*
Chicken beta actin gene: *actb*
Beta actin protein: ACTB

**Quantitative PCR:** If the quantitative PCR method has been used, the related section in Materials and Methods must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

The following information must be provided in the section:

Protocol for DNA/RNA extraction, including quantification and determination of purity;

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components;

qPCR: sequence of forward and reverse primers, probes, amplicon size, accession number of Genebank; thermocycler parameters (i.e. denaturation, annealing and extension steps, number of cycles, melting curves); validation of PCR products; non-template controls for reverse transcription and qPCR should be included in all reactions; and

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***Example piece of text and reference list :***

An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

***References for the above example:***

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan Goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

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## *Tables*

Please submit tables as individual files and editable text and not as images. Place all table notes below the table body. Each table should have a title which is followed by explanation of results shown in the table. Use of vertical rules must be avoided. Tables should be self-explanatory, and clearly arranged. Tables should provide easier understanding and not duplicate information already included in the text or figures. Each table should be typewritten with double spacing on a separate file and numbered in order of citation in the text with Arabic numerals. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Explain any non-standard abbreviations in a footnote to the table.

### *Figures*

Figures must be submitted in individual files (format: TIFF, Dimensions: Width: 789 – 2250 pixels at 300 dpi Height maximum: 2625 pixels at 300 dpi, Resolution: 300 – 600 dpi, file size: less than 10 MB, Text within figures: Arial or Symbol font only in 8-12 point). The text and other labels should be placed in the figure as un-compressed layers. Each figure should have a title which is followed by explanation of results shown in the figure. Figures should be numbered in order of citation in the text with Arabic numerals.

For the use of bar diagrams the following publication should be consulted:

Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015; 13(4):e1002128.

The bar diagrams should be provided in color and in a well-designed and professional format. Please do not use different shades of gray. The axes of diagrams should have titles and units. Also, the source file of the image (Excel etc.) should be provided for typesetting.

Illustrations should be numbered as cited in the sequential order in the text, with a legend at the end of the manuscript. Color photographs are accepted at no extra charge. The editors and publisher reserve the right to reject illustrations or figures based upon poor quality of submitted materials.

If a published figure is used, the publisher's permission needs to be presented to the office, and the figure should be referenced in its legend.

### *Use of Italics*

Gene symbols, Latin terms (i.e. *in vivo, in vitro, ex vivo, in utero, in situ*, and etc.) and species scientific names (using the binomial nomenclature), should be typed in italics, while the first letter of the genus name must be capitalized (i.e. *Homo sapiens*).

## PUBLICATION ETHICS

Iranian Journal of Veterinary Science and Technology is aligned with COPE's (Committee on Publication Ethics) best practice guidelines for dealing with ethical issues in journal publishing and adopts the COPE guidelines. The journal members (editor, editorial board and the journal manager) have agreed to meet the purposes and objectives of the Journal.

## Ethical guidelines for authors:

### *Authorship Criteria*

IJVST requires authors to confirm that they and their co-authors meet all four criteria for authorship based on the guidelines of The International Committee of Medical Journal Editors (ICMJE) (verbatim as follows):

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The section "Author Contributions" in the manuscript should illustrate and clarify who contributed to the work and how. If a contributor does not meet all four above criteria should be acknowledged in the "Acknowledgements" section of the article.

### *Author agreements and conflict of interest*

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# Publication Ethics



### *Ethical guidelines for Peer reviewers*

Iranian Journal of Veterinary Science and Technology (IJVST) follows and adheres to COPE Ethical Guidelines for Peer Reviewers. IJVST peer reviews all submitted manuscripts with contents in the scope of the journal. The process has been explained in the section "Peer Review Process".

### *Ethical guidelines for Editor*

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## PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

### *Initial assessment*

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

### *Initial screen*

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

### *Peer review (double-blind)*

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

### *Final Decision*

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.


# Iranian Journal of Veterinary Science and Technology

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