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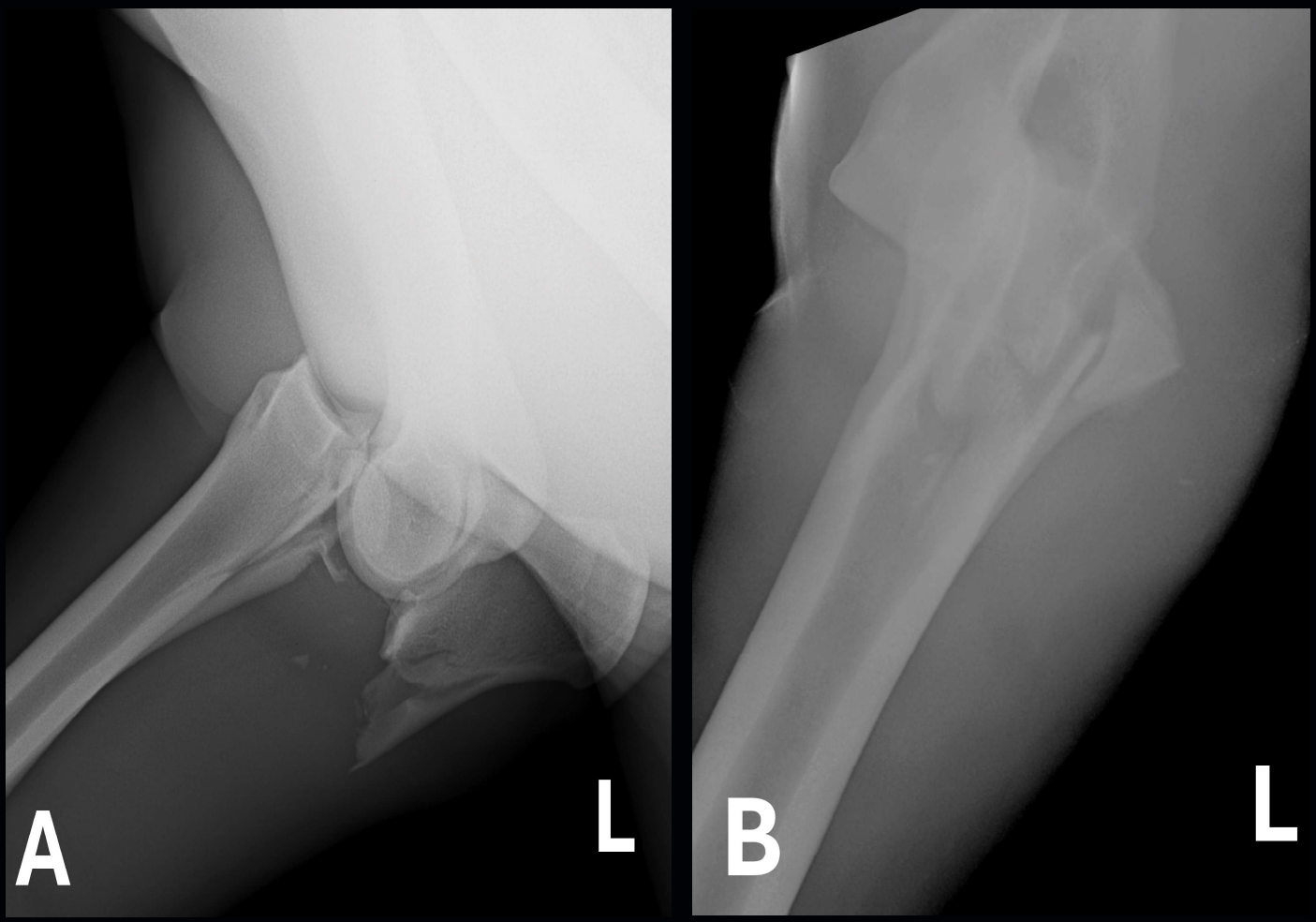

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In the Name of God, the Compassionate, the Merciful

Iranian Journal *of* Veterinary Science and Technology

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ON THE COVER

A Type I Monteggia fracture in a mule is characterized by spiral, comminuted fractures of the proximal ulna accompanied by complete anterior luxation of the humeroradial joint (see page 72).

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RESEARCH ARTICLE

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Kocuria rhizophila As the Etiological Agent Infecting Rainbow Trout (*Oncorhynchus mykiss Walbaum, 1792*) in Iran

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ABSTRACT

An natural acute disease outbreak occurred in two rainbow trout farms located in northern Iran. Affected fish exhibited clinical symptoms, including ocular opacity, scale desquamation, skin necrosis, superficial to deep lesions on the dorsal and lateral body surfaces, loss of caudal peduncle tissue, mild exophthalmia, skin melanization, hepatic hemorrhage and paleness, excessive mucus accumulation in the abdominal cavity, renal paleness, and intestinal infection. Following biochemical analysis of the bacterium, gene sequencing was conducted, leading to the identification and registration of a strain known as *K. rhizophila* TMU97910 in the NCBI gene bank. After the elucidation of the bacterium's identity, salinity tolerance, and antibiogram tests were conducted. The results demonstrated that the strain exhibited complete resistance to salinity, allowing it to thrive in a wide range of salinity conditions. In antimicrobial assays, the isolate showed resistance to Fosfomycin, while remaining sensitive to Florfenicol, Doxycycline, Oxytetracycline, Enrofloxacin, Sulfamethoxazole/Trimethoprim, Erythromycin, Gentamycin, and Kanamycin. To investigate the behavior of this bacterium in rainbow trout, pathogenicity, LD_{50}, and histopathology analyses were conducted in three replicates. Pathogenic symptoms in trout remained consistent. Histopathological examination of the liver, kidney, spleen, and gills revealed extensive tissue damage caused by this strain, including necrosis, hemorrhage, degeneration, and tissue wounds. Subsequently, bacterial recovery was achieved, and negative results for the detection of other pathogens confirmed that Kocuria rhizophila was responsible for the disease outbreak.

Keywords

Kocuria rhizophila, Rainbow trout, Pathogenicity, histopathology, antibiogram test, Susceptibility

Abbreviations

No abbreviations

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Introduction

One of the prominent species cultured in the aquaculture industry is Rainbow trout (*Oncorhynchus mykiss*). Despite the increasing investment in its cultivation to produce and supply aquatic food products, rainbow trout is susceptible to a variety of bacterial infections, leading to significant production losses due to morbidity and mortality. Prompt identification and reporting of emerging infectious diseases are crucial to ensure optimal biosecurity practices that support the development of health and welfare in farmed fish, as well as the promotion of global product placement.

Understanding the inhibiting factors that impact the progression of aquaculture is crucial. Therefore, this essay delves into the outbreak of a disease in rainbow trout farms situated in northern Iran, which was documented in November 2018. Affected fish exhibited symptoms such as hemorrhagic septicemia, extensive wounds on the dorsal and lateral areas, and lesions on the caudal peduncle, r in some cases leading to the complete exposure of the vertebral column. Additionally, some parts of the skin were detached from the underlying tissue. Reports from the affected sites also indicated occurrences of exophthalmia, skin melanization, ocular opacity, and elevated mortality rates.

Preliminary diagnosis based on both clinical observations and biochemical analyses suggested a bacterial etiology, indicating the emerging pathogen Kocuria spp. Members of the Kocuria belong to the family Micrococcaceae, within the order Actinomycetes [1]. Based on the phylogenetic reclassification of the genus Micrococcus using 16S rDNA sequencing and chemotaxonomic data, the genus was found to be heterogeneous, with its species distributed across five distinct clusters. Consequently, only *M. luteus* and *M. lylae* were retained within the emended genus Micrococcus, while three species *M. roseus*, *M. varians,* and *M kristina* were transferred to the novel genus Kocuria [2]. These Gram-positive coccoid bacteria whose main habitat is soil were first isolated and reported from the rhizoplane of Typha angustifolia [3].

Overall, The aforementioned information serves as the basis for asserting that a recent discovery has identified a specific species within the Kocuria genus, Kocuria rhizophila, as an "emerging pathogen" responsible for causing illness in brown trout and farmed rainbow trout in Poland [4]. It is clear that there is limited available data regarding the impact of this microorganism on natural disease outbreaks in aquaculture settings. Therefore, the present study

aims to investigate the prevalence, virulence factors, histopathological features, and control measures of an emerging disease caused by *Kocuria rhizophila* in rainbow trout farms in Iran, through the isolation and identification of the bacterium from regional aquaculture facilities.

Results

Fish Sampling During a Natural Disease Outbreak

Moribund rainbow trout exhibited a range of external clinical signs consistent with systemic bacterial infection. These included hemorrhagic septicemia, deep ulcerative lesions on the dorsal and lateral body surfaces, and lesions localized to the caudal peduncle. Additional clinical manifestations included exophthalmia, skin melanization, and ocular opacity. Internally, the fish displayed renal opacity, liver congestion, and intestinal inflammation.

Bacteria Isolation, Identification, and Morphological Characteristics

Regardless of the agar type used, colony growth was completed at 22°C after approximately 48-72 hours and at 37°C within 24 hours. The bacterial colonies were characterized as non-mucoid, yellow pigmented, convex circular, and umbonate with entire margin. No hemolytic activity was observed on blood agar. The cultures were identified as Gram-positive cocci, exhibiting positive oxidase and catalase reactions. The isolates were non-motile, and exhibited a non-fermentative metabolic profile, as indicated by the TSI test, which showed no gas or H2S production. Additionally, the bacterium did not metabolize tryptophan, mannitol, or citrate, and no glucose fermentation was detected.

Bacterial DNA extraction and identification using PCR and 16SrRNA sequencing

A distinct 1500 bp amplicon was observed in the eubacterial PCR assay. However, 16S rRNA sequencing yielded a partial gene sequence of 1394 bp, which was subsequently submitted to the GenBank database. Sequence analysis of the 16S ribosomal RNA confirmed the isolate as Kocuria rhizophila strain TMU97910. The strain name was officially registered in the National Center for Biotechnology Information (NCBI) database. The nucleotide sequence has been assigned the accession number MW947584, and the complete genome sequence, and interpretation of *K. rhizophila* TMU97910 can be accessed via the following NCBI link: https://www.ncbi.nlm.nih.gov/nuccore/MW947584. The phylogenetic tree is presented in Fig 1.

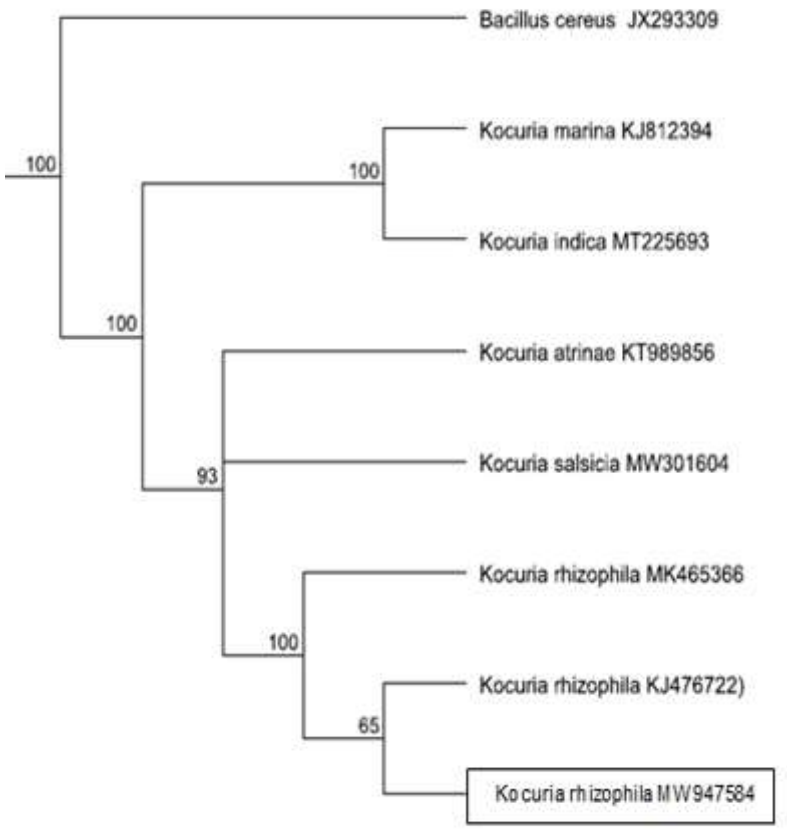


Figure 1.
The Kocuria rhizophila phylogenetic tree based on 16SrRNA sequence

Salinity tolerance test

The strain *K. rhizophila* TMU97910 exhibited robust growth across all NaCl concentrations, as indicated by viable colony counts. Conversely, no growth was detected in the negative control.

Antibiogram Testing

The antibiotic susceptibility profile of *K. rhizophila* strain TMU97910 is presented in Table 1. The strain demonstrated sensitivity to several antibiotics, including Florfenicol, Enrofloxacin, Oxytetracycline, Doxycycline, Sulfamethoxazole/Trimethoprim, Erythromycin, Gentamycin, and Kanamycin. However, the strain exhibited resistance to Fosfomycin.

Table 1.
Antibiogram test of Kocuria rhizophila TMU97910

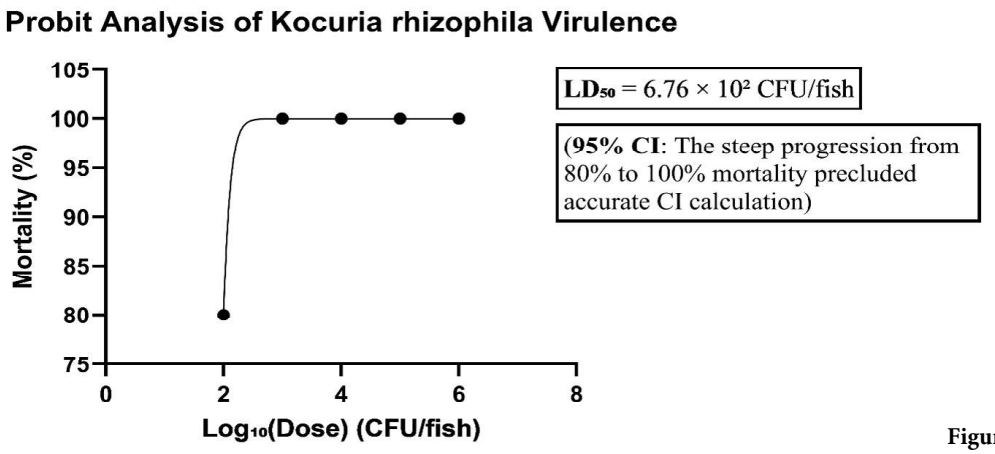
Kocuria rhizophila	Disc content	Antimicrobial agent
40 mm- S	30 µg	Florfenicol
37 mm- S	1.25/23.75 µg	Trimethoprime/sulfamethoxazole
40 mm- S	10 µg	Gentamicine
21 mm- S	30 µg	Kanamycine
35 mm-S	30 µg	Oxytetracycline
40 mm-S	30 µg	Doxycycline
0 mm-R	20 µg	Fosfomycin
35 mm-S	5 µg	Enrofloxacin

Pathogenicity and LC_{50}

The mortality of rainbow trout began in all fish groups exposed to the bacterium within 48 hours post-injection. The mortality patterns were consistent across all concentrations during the initial four days. At the lowest concentration (102 CFU/fish), mortality reached 60%. Subsequently, after seven days of stability, mortality increased rapidly to 80% by day 11. In the 10^3 CFU/fish group, mortality also reached 60%, remained stable for six days, and then increased sharply to 100% by day 11 post-infection. Higher concentrations (10^4, 10^5, and 10^6 CFU/fish), resulted in rapid mortality within the first three days, reaching 60%, 80%, and 40% respectively. After one day of stability from day 4, there was a rapid increase in mortality exceeding 100%. The results of the probit analysis and the survival plot are presented in Figures 2A and 2B, respectivel.

The experimental challenge study revealed typical clinical signs observed in moribund rainbow trout exposed to the bacterium. Affected fish exhibited skin damage, necrotic areas leading to tissue damage of the caudal peduncle, focal ulcers on the dorsal and lateral body surfaces, ocular opacity, and pale liver and kidney.

A



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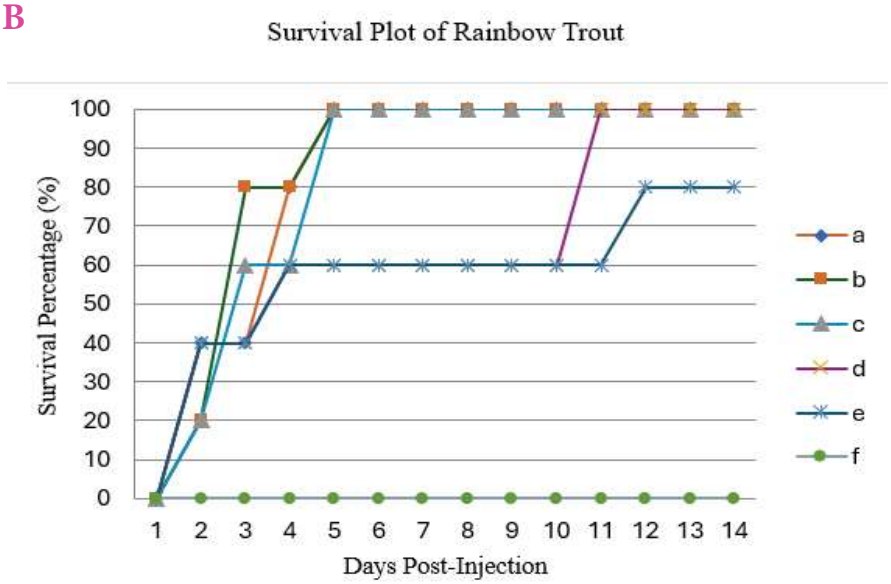

Figure 2.
2A. Probit analysis for determination of LD_{50} in rainbow trout challenged with Kocuria rhizophila TMU97910.
2B. Kaplan-Meier survival plot of rainbow trout challenged with Kocuria rhizophila. Statistical significance was determined using Log-rank test ($p < 0.0001$).

Histopathology

Histopathological results are presented in Fig 3. Re-isolation of the bacterium from the liver, kidney, spleen, and gills, followed by biochemical tests, in accordance with Koch's hypothesis, verified the presence of *K. rhizophila* in these organs. The histopathological changes in the rainbow trout (B, E, H, K) injected with *K. rhizophila* were examined. The histological alterations in the kidney demonstrated tubular degeneration, severe lesions, and necrosis (B). The liver illustrated cellular necrosis, hypertrophy, congestion, hemorrhage, sinusoidal dilation, and the presence of pyknotic nuclei. The gills showed necrosis, edema, hypertrophy of mucous cells, curvature, clubbing of the tips, shortening of secondary lamellae, and epithelial individuation (H). The spleen tissue of the rainbow trout illustrated hemosiderin, necrosis, white pulp, and red pulp (K).

Discussion

To address the emerging threat of *Kocuria rhizophila* in rainbow trout aquaculture, this investigation focused on determining the occurrence, virulence attributes, histopathological damage, and practical control strategies, drawing upon bacterial strains successfully isolated from affected farms across Iran. The present study successfully fulfilled Koch's postulates, confirming that the strain identified as Kocuria rhizophila TMU97910 is capable of inducing disease under experimental conditions, similar to the natural disease observed in rainbow trout. Although this bacterium was not considered a pathogen to farmed fish species before the first report [4], the results from the current study, similar to Pekala's report, support the emergence of *K. rhizophila* as an emerging fish pathogen. In the current study, the disease emerged in an acute form in the investigated farms. The naturally infected fish presented a broad range of clinical signs, including exophthalmia, melanization, skin lesions,

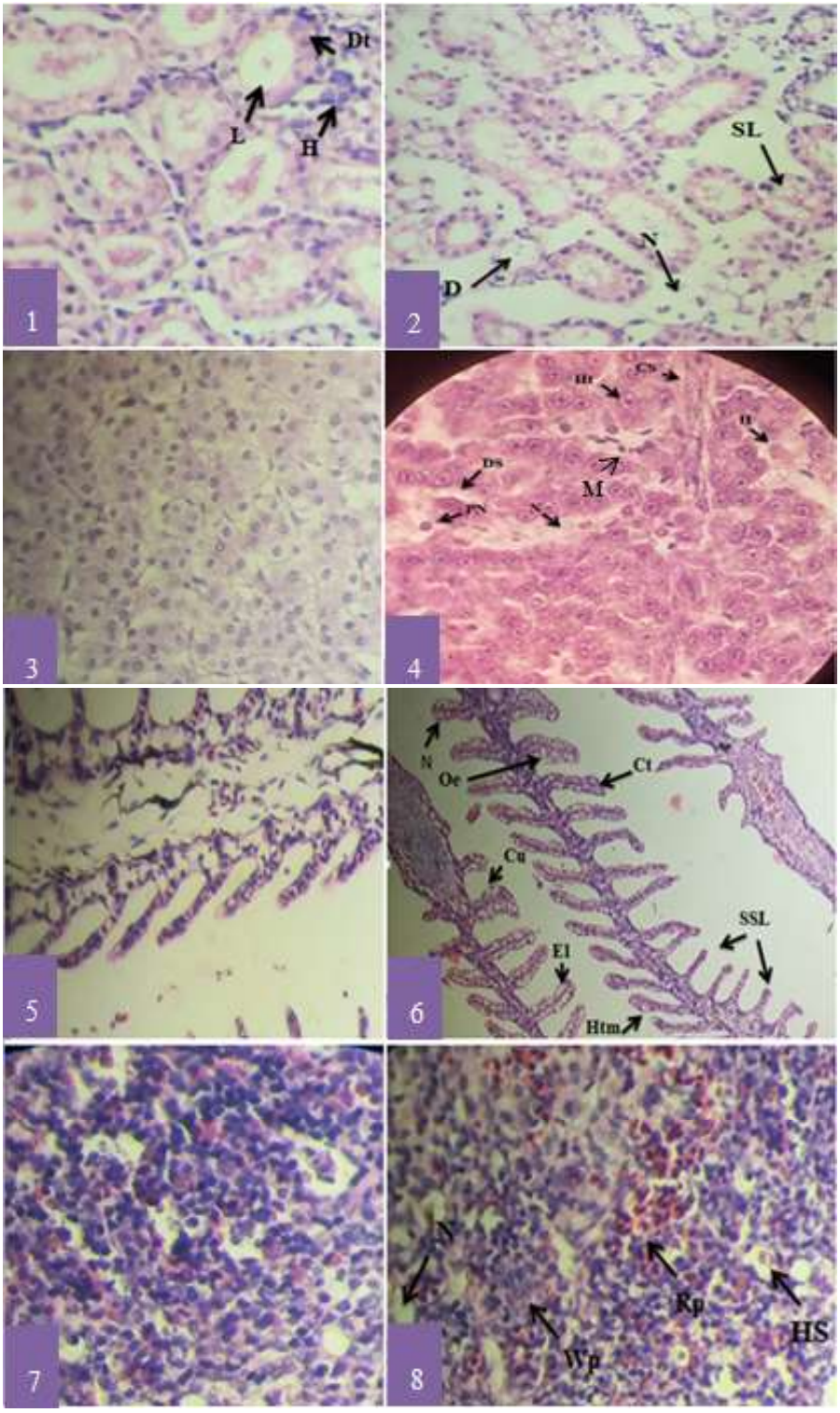

Figure 3.
Histopathological changes in rainbow trout due to Kocuria rhizophila infection were observed in various tissues (2, 4, 6, 8). The histological examination of the kidney from the infected group revealed tubular degeneration, severe lesions, and necrosis (2), whereas the liver exhibited melanomacrophages, necrosis, hypertrophy, congestion, hemorrhage, sinusoidal dilatation, and pyknotic nuclei (4). Similarly, the gills showed necrosis, edema, hypertrophy of the mucous glands, curvature, clubbing of the tips, shortening of secondary lamellae, and epithelial individuation (6). The spleen tissue of the infected rainbow trout displayed hemosiderin, necrosis, white pulp, and red pulp (8). Abbreviations: (M) Melanoma macrophages; (H) hematopoietic tissue; (L) tube lumen; (Dt) distal tubules; (D) degeneration of tubules; (SL) Severe lesions; (N) necrosis; (Ht) Hypertrophy; (Cs) Congestion; (H) Hemorrhage; (Ds) Dilatation Sinusoidal; (PN) Pyknotic Nuclei; (HS) Hemosiderin; (Oe) Oedema; (Htm) Hypertrophy of the mucous glands; (Cu) Curvature; (Ct) Club tip; (SSL) shortening of secondary lamellae; (EI) Epithelial individuation; (Wp) White pulp; (Rp) Red pulp; Fibroblast. Stained with hematoxylin and eosin (x400)

necrotic tissue erosion, ocular opacity, superficial to deep wounds on the body tissue, pale liver and gills, along with intestinal inflammation. Notably, a similar spectrum of clinical symptoms was observed in rainbow trout infected by *K. rhizophila* naturally and artificially (experimental challenge).

Prior to 2016, data in the NCBI Gene Bank database did not document the isolation of *K. rhizophila* from diseased fish. The first report of its pathogenicity in aquaculture industry was recorded in 2018 in Poland [4].

In the current study, *K. rhizophila* strain TMU97910 required an incubation period between 48 to 72 h when grown on artificial media incubated at 22 or 37°C. This was in agreement with previous studies by Savini [5] and Pekala [4] on strains of *K. rhizophila*.

Another remarkable feature of the genus Kocuria is its halotolerant nature. Several species, including K. kristinae, *K. rhizophila*, and K. marina, have been reported to tolerate sodium chloride concentrations exceeding 10% [3; 6]. In the present study, the strain *K. rhizophila* TMU97910 could grow at all salinity ranges tested under in vitro conditions, aligning with previous studies for members of this genus. This feature demonstrates that the strain has the potential to cause disease not only in brackish and freshwater fish but also in saltwater fish. In other words, the strain showed to survive in saline environments from zero to saturation effortlessly.

The antibiotic susceptibility profile of Kocuria species remains incompletely understood. Although there are case reports of antimicrobial susceptibility test results, they are insufficient to determine the exact susceptibility of Kocuria species [7]. Limited data is available on the antibiotic sensitivity of emerging fish pathogen *K. rhizophila*. For example, Pakala et al. [4] observed susceptibility of their *K. rhizophila* strains to doxycycline and amoxicillin/clavulanate, whereas resistance to norfloxacin was observed in a study where *K. rhizophila* was isolated from the blood of a child with sepsis [8]. Another study reported resistance of *K. rhizophila* isolated from a sick child's blood to ciprofloxacin and erythromycin [9]. Meletis [10] found that Kocuria isolated from a continuous ambulatory peritoneal dialysis (CAPD) patient was resistant to levofloxacin. Lee [11] highlighted the importance of Kocuria in nosocomial infections, suggesting that Kocuria species should be considered potential pathogens in immuno-compromised patients, particularly in intensive care and neonatal units. They reported that over 50% of patients suffered invasive infections with Kocuria as a secondary disease. Studies on drug resistance genes have indicated that DnaE2, a component of replicating DNA polymerases, may contribute to drug resistance in Mycobacterium tuberculosis [12]. The genome of *K. rhizophila* DC2201 also contains a dinB homolog (KRH_14930) that may encode the Y family-susceptible DNA polymerase IV, potentially leading to drug resistance [13]. In the present study, *K. rhizophila* exhibited resistance only to fosfomycin while remaining sensitive to a broad range of antibiotics, including florfenicol, doxycycline, oxytetracycline, enrofloxacin, sulfamethoxazole/trimethoprim, erythromycin, gentamicin, and kanamycin, which could aid in controlling and treating diseases caused by this strain.

It is important to note that the identification and confirmation of pathogenic agents require pathogenicity studies. In other words, in the case of potential emerging or novel diseases, pathogenicity assessment are essential to confirm whether the potential pathogen is the etiological agent responsible for the observed disease outbreak. In the presented study, the bacterium was introduced via injection, which is a common method for determining etiology but is also the most unnatural, as it bypasses the host's immune response, providing a better chance for the bacteria to spread and cause disease. Therefore, studies on the natural exposure of fish to bacteria are necessary to determine disease risk. However, the bacterial strain recovered from natural outbreaks in the farms not only caused high mortality in rainbow trout but also induced clinical signs of the disease.

As no accessible results were available to determine the LD_{50} of this bacterium in rainbow trout, we investigated the LD_{50} in *O. mykiss*. According to the linear graph, the mortality trend increased rapidly in the first three days and reached 100% by the 14 day in all groups except at a concentration of 10^2 CFU/fish. The LD_{50} at 72 hours was 5×10^9 CFU/fish. Therefore, these results serve as a warning to the aquaculture industry regarding the significance of this bacterium in causing disease in these fish.

Histopathological analysis remains the primary diagnostic tool available for fish disease, while other methods are used for laboratory confirmation of pathogen identification. In the present study, the histological changes in the kidney demonstrated tubular degeneration, severe lesions, and cellular necrosis. The liver showed accumulation of melanomacrophages, necrosis, hypertrophy, congestion, hemorrhage, sinusoidal dilatation, and pyknotic nuclei [14]. Haaparanta [15] reported that pigments within melanomacrophage centers possess bactericidal properties, which may explain the accumulation of melanomacrophages during infection. Additionally, as a result of the entry of foreign agents into the fish body, phagocytic cells accumulate at the site and continue to cleanse the dead agents and cells after their elimination [16].

In the gills, necrosis, edema, mucous glands hypertrophy, club tip, shortening of secondary lamellae, epithelial individuation, and curvature were observed. The shortening of secondary lamellae in gills may lead to reduced respiratory gas exchange, as reported in the study by Ostaszewska [17]. Edema and epithelial individuation in rainbow trout are likely associated with osmotic imbalance, as previously described by Al-Bairuty [14]. Moreover, hypertrophy is a compensatory reaction to decrease the absorption of environmental factors. Overall, these mechanisms increase the distance between the external environment and the blood, serving as an obstacle to the entrance of foreign agents. The decrease in surface area causes a reduction in respiration, as recorded in the study by Camargo [18].

In the spleen, the presence of necrosis, hemosiderin deposits, and white and red pulp were observed. The presence of hemosiderin in the rainbow trout infected by *K. rhizophila* at a concentration of 106 CFU/fish is due to b-hemolysin, which performs hemolysis in the fish's body and causes the deposition of hemosiderin, as reported in the study by Abdelhamed [19].

The areas of necrosis are the most significant lesions created in all four tissues. Histological changes might be toxin-mediated bacterial infections, evidenced by the level of hemorrhages and cellular necrosis, similar to descriptions reported by Abdelhamed [19]. Other studies have indicated that when fish are exposed to toxic substances, the gills present lesions such as necrosis, hypertrophy, and hypertrophy of the mucous glands [20; 21; 22].

In a study by Dong [23], it was stated that histopathological changes induced by gram-negative and positive bacteria are often similar, including necrosis, infiltration of inflammatory cells, degeneration, hydrolysis, fat degeneration, and hemorrhage. According to them, kidney damage in fish was observed with almost all isolates of gram-positive bacteria. It is evident that serious damage has been done to the tissues by this strain.

Despite the documentation of Kocuria species in various countries, there has been no prior documentation of their pathogenicity in the Iranian aquaculture industry. This study revealed that *K. rhizophila* is an emerging fish pathogen capable of causing disease in rainbow trout, representing the first reported rainbow trout infection by this strain in Iran. Given the significance of this disease in the aquaculture industry, this study conducted pioneering research on the histopathology, pathogenicity, and LD_{50} of this bacterium. Additionally, as it is an emerging disease without a specific designation, the disease caused by *K. rhizophila* can be termed "Kocuriasis."

Materials and Methods

Fish sampling and bacteria isolation

In November 2018, a total of 20 moribund rainbow trout exhibiting clinical manifestations, consistent with those described by Pekala [4], were collected from two aquaculture facilities in Amol, northern Iran. The fish were transported to the laboratory of the Faculty of Marine Sciences at Tarbiat Modares University (Noor), under aeration at a temperature of 17°C for 45 minutes. Upon arrival at the laboratory, the fish underwent a gross examination to assess clinical signs of disease, and samples were collected for bacterial isolation following the methodologies outlined in the study by Pekala [4].

Prior to sampling, the fish were rinsed with physiological saline (0.9% NaCl, Merck) to eliminate any external contaminants. Subsequently, aseptically, tissue samples from the kidney, liver, and spleen were cultured on Blood Agar plates (BA; 5% sheep blood, Ibresco), Trypticase Soy Agar (TSA, Ibresco), and Nutrient Agar (NA, Liofilchem). The samples were then incubated at 22°C for 24-72 hours until visible colonies appeared. Blood culturing (from the caudal vessel) was conducted following the protocol outlined by Weinstein [24]. Briefly, 0.1 mL of broth was spread-plated onto BA, TSA, and NA media and incubated at 22°C for 24-72 hours. Daily monitoring of all samples continued for a maximum of 72 hours, with detailed descriptions of colony growth and morphology. Purification was carried out as necessary. Subsequent subculturing of a single colony on BA was performed at both 22°C and 37°C for 24-48 hours. Pure cultures were then preserved at -80°C in Tryptic Soy Broth (TSB, Ibresco) supplemented with 15% glycerol (Merck) [4].

Bacteria identification and morphological characteristics

Morphological and biochemical characterizations were conducted according to the methods outlined in Buller [25]. Phenotypic and biochemical reactions were assessed through a series of tests, including Gram staining, oxidase and catalase tests, hemolysis analysis, motility examination, Indole test, iron utilization test, sodium thiosulfate test (SIM), growth on Mannitol Salt Agar (MSA), Triple Sugar Iron (TSI) test, MacConkey agar (MAC) growth, Methyl Red/Voges-Proskauer (MRVP) test, and Citrate utilization test as described by Buller [25]. Each sample was incubated at either 22°C or 37°C for a period of 48-72 hours, with positive and negative controls set at each temperature. Subsequently, the results of these tests were recorded.

Bacterial DNA extraction and identification using PCR and 16SrRNA sequencing

Genomic DNA was extracted from the isolate utilizing the methodology outlined in the commercially accessible Sinagen kit (Iran). In summary, a single colony was cultured into 100 ml of Tryptic Soy Broth (TSB) for 24 hours at 37°C, and bacterial DNA was isolated using a digestion buffer comprising Sodium Dodecyl Sulfate (SDS), sodium Chloride-Tris-EDTA (STE) buffer, and proteinase K. The purification process involved two stages of extraction with Phenol, Chloroform, and Isoamyl alcohol (25-24-1), followed by DNA precipitation with absolute ethanol. Subsequently, the DNA was eluted in 50 µL of sterile distilled water. The sample underwent 16SrRNA PCR analysis as per the techniques delineated by Sanger [26] employing the primer set: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) [26]. The PCR was carried out in a PeQlab thermocycler (Germany), with under the following conditions: initial denaturation at 95°C for 15 minutes, followed by 35 cycles comprising denaturation at 95°C for 30 seconds, annealing at 72°C for 25 seconds, and extension at 72°C for 10 minutes.

Subsequently, 5 µl of the product was visualized on a 2% agarose gel in TBE 1X buffer supplemented with Greenview fluorescent dye. The PCR product underwent purification using a Column RNA isolation kit and was then sent for sequencing to Transferred (BJI Company; China). A comparison between the sequence of the isolate and existing entries in Gen Bank was conducted, and a phylogenetic tree was constructed utilizing MEGA 7.0 software.

Salinity tolerance test

Salinity tolerance was evaluated using a standard bacterial growth assay as previously described [3]. A single colony was aseptically inoculated into 10 ml of Tryptic Soy Broth (TSB) with varying NaCl concentrations (5% - 40% w/v). The cultures were incubated in a rotary shaker (150 rpm) at 22°C for 24 hours. Bacterial growth (turbidity) was quantified using a spectrophotometer (JENWAY 6305 UV/VIS) at OD_{600} [27]. After 24 hours, 100 µl of the culture was aseptically spread on Tryptic Soy Agar (TSA) plates to confirm cell viability and then incubated at 22°C for another 24 hours.

Antibiogram test

Antibiogram tests were conducted using the Kirby-Bauer disc diffusion assay, following the procedures outlined in CLSI (2006). In summary, a bacterial suspension was prepared in sterile physiological saline and adjusted to match the 0.5 McFarland standards. Lawn cultures were established by aseptically applying swabs saturated with the bacterial suspension onto clean Muller Hinton agar (Ibresco) plates. Subsequently, the plates were allowed to air dry at room temperature for 5 minutes before antibiotic-impregnated paper discs (procured from Padtan Teb) were carefully positioned on the agar and then incubated at 22°C for 24 hours. Each agar plate accommodated a maximum of 5 discs. The zones of inhibition were measured, and the findings were categorized as sensitive (≥16 mm), intermediate (12–15 mm), or resistant (≤11 mm) based on the diameter of the inhibition zones [28]. The antibiogram tests included: Florfenicol (FF-30 µg), Enrofloxacin (NFX-5 µg), Oxytetracycline (T-30 µg), Doxycycline (D-30 µg), Sulfamethoxazole/Trimethoprim (SXT-1.25 / 23.75 µg), Erythromycin (E-15 µg), Gentamycin (GM-10 µg), Kanamycin (K-30 µg), and Fosfomycin (Fos-20 µg).

In-vivo Fish Challenge

To assess the pathogenicity, an in vivo challenge was conducted on rainbow trout. The fish (130-145 g) were obtained from a local farm in Mazandaran province, transported, and placed in 100-liter aerated tanks under a 12-hour light/dark cycle, with a water temperature of 17°C, oxygen levels at 8 mg/l, and a pH of 7.2. Throughout the 14-day acclimation period, the fish were fed twice daily with a standard commercially extruded feed, and fifty percent of the water was exchanged daily.

To enhance the bacterial virulence, individual fish (n=3) were injected with 1.2×10^8 CFU/fish. The bacterium was then isolated from the moribund fish and subsequently identified. This passaged strain was selected for the challenge and subsequent LD_{50} studies.

For LD_{50} determination, fish were randomly distributed into experimental groups (n = 5 per tank). There were six treatment groups where each fish were exposed to the bacterium through intraperitoneal injection of a 0.1 ml volume of the bacterial suspension at concentrations of $10^2/10^3/10^4/10^5/10^6$ CFU/fish. The final treatment group served as a control, where fish were treated in the same manner but did not receive the bacteria; instead, the control fish were injected with 0.85% (w/v) saline. The LC_{50} (96h) was calculated using Probit Analysis with a 95% confidence interval [29].

For the bacterial challenge, the bacterium was cultured in TSB for 24 hours at 22°C. The cells were then harvested by centrifugation and resuspended with sterile saline to reach a specific optical density (OD_{600}). The concentration of the bacterium resulting in more than 50% mortality was confirmed during the LD_{50} stage using viable colony counts, following the method described by Miles and Misra [30].

Each fish in treatment group one was exposed to the bacterium through intraperitoneal injection of a 0.1 ml volume of the bacterial suspension. Fish in treatment group two received a similar volume of sterile 0.85% (w/v) saline via intraperitoneal injection, as outlined by Alghabshi [31]. The fish were monitored daily for 14 days, with mortality and morbidity recorded every 24 hours. Any moribund fish was removed and sampled for histopathology (limited to moribund fish only), with bacterial recovery and identification conducted as previously described.

The fish were observed daily for any clinical signs of disease, including reduced feeding and behavioral changes. Samples were collected for bacterial recovery and histopathology as outlined. These trials were conducted to fulfill Koch's postulates.

Histopathology

Tissue samples from the gills, liver, spleen, and anterior kidney were collected from three fish per treatment group (when they exhibited immobility and weakened operculum performance). Samples were were aseptically extracted and processed for histopathological analysis, following the procedures outlined in the study by Chen [13]. Initially, all tissues were immersed in 10% formalin (Sigma-Aldrich) for 24 hours for fixation followed by processing using a Tissue Processor (DS2080/H, Iran) and Tissue Float Machines (Labtron, Iran), followed by embedding in wax. The tissue sections were sliced to a thickness of 5 micrometers using a Microtome Accu-Cut (Accu-Cut® SRM™ 200 Rotary Microtome) and subsequently stained with hematoxylin and eosin (HE) as described by Chen [32]. The tissue samples affixed to microscope slides were examined under a light microscope at magnifications of up to 100×, equipped with a camera (Optika, B-293, Italy) for image capture, in accordance with the methodology detailed by Legario [33].

Authors' Contributions

Amir Hossein Smiley , Mohammad Reza Kalbassi conceived and planned the experiments. Nazila Yeganeh , Amir Hossein Smiley carried out the experiments. Nazila Yeganeh, Amir Hossein Smiley, Mohammad Reza Kalbassi, Margaret Crumlish contributed to the interpretation of the results. Nazila Yegane, Amir Hossein Smiley took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Autologous platelet-rich plasma promotes regeneration of ethanol-induced endometrial damage in a canine model

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ABSTRACT

Thin and damaged endometrium represents a significant challenge in managing pregnancies, and may contribute to postpartum uterine disorders, recurrent miscarriages, and other complications. Although various treatments have been proposed, their effectiveness remains inconsistent. Recently, scientists have turned their attention to platelet-rich plasma (PRP) for its growth factors and cytokines in human medicine. However, its potential for reconstructing endometrial issues in canine models has not yet been adequately investigated. Therefore, the present study aimed to investigate the therapeutic properties of PRP on ethanol-induced endometrial damage in dogs. Twelve healthy adult mixed-breed dogs were randomly divided into four groups: group E received 95% ethanol in uterine horns to create a model of endometrial damage; group E+NS received saline 72 hours post-injury; group E+PRP received PRP 72 hours post-injury; and group NS+PRP was initially given normal saline and PRP 72 hours later. The results showed significant improvements in endometrial thickness, epithelial repair, and uterine gland density in the E+PRP group compared with the E group ($P < 0.05$). The E+NS group did not show significant differences in comparison to the E group. The results showed a decrease in bleeding and necrosis severity in the E+PRP group. Additionally, hyperemia and hemosiderosis levels in the E+PRP, E+NS, and E groups were similar. In conclusion, the utilization of PRP was successful in the treatment of ethanol-induced endometrial injury in dogs. These findings suggest that PRP may represent a promising regenerative therapy for thin endometrium and related reproductive disorders.

Keywords

Platelet-rich plasma, thin endometrium, regenerative medicine, histology, dogs

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Abbreviations

ANOVA: analysis of variance
CTGF: connective tissue growth factor
E: ethanol
EGF: epidermal growth factor

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Introduction

The endometrium plays a vital role in the female reproductive system. Structurally, it consists of blood vessels, glands, epithelial tissue, and stroma. Previous studies have indicated that alterations in uterine tissue composition and damage can significantly disrupt the normal physiological cycle, affecting embryo implantation and overall pregnancy outcomes [1-3]. Moreover, endometrium thickness is important, as it affects implantation of embryo, delivery, and fetal birth weight [4-8]. Clinical and experimental evidences have highlighted that variations in endometrial thickness can significantly influence embryo survival rates after intrauterine insemination, with thinner endometria correlating with lower survival rates [9, 10].

The pathogenesis of thin endometrium remains incompletely understood and is likely multifactorial. Several mechanisms have been proposed, including increased cellular senility, elevated collagen deposition, and decreased expression of genes, all derived from models of thin uterine disease, as well as inflammatory atrophy and immune dysfunction observed in veterinary endometritis cases [11]. As a result, a range of therapies has been developed to counteract these pathogenic pathways. For instance, the efficacy of vaginally administered sildenafil has been examined in patients who have experienced failure in in vitro fertilization (IVF) as a result of endometrial dysplasia [12]. Additionally, other treatments such as estrogen, long-acting aspirin administered in low-doses, and intrauterine infusion of granulocyte colony-stimulating factor (G-CSF) have been employed [13, 14]. Although some of these interventions have shown promising results, the effectiveness of these therapies remains inconsistent, and an optimal treatment approach for thin endometrium has not yet been established.

Platelet-rich plasma (PRP) is known for its high concentration of platelet activators that stimulate cytokine and growth factor activity. Evidence suggests that intrauterine injection of PRP can enhance endometrial growth and implantation. PRP contains a greater number of platelets compared to general circulation, which allows it to promote proliferation and repair through a rich supply of various cytokines and growth factors, such as transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), connective tissue growth factor (CTGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and interleukin-8. Although platelet transfusion is applied to an increasing extent in various medical fields including osteoarthritis, nerve injury, and plastic surgery, its use in gynecological and obstetric conditions is still limited [15, 16]. To date, no published in vivo or in vitro studies have specifically evaluating the effect of PRP growth factors on the canine endometrium. Consequently, the objective of this research was to evaluate the therapeutic impact of PRP on the treatment of thinned endometrium in a canine model.

Abbreviations-Cont'd

ELISA: enzyme-linked immunosorbent assay
FGF: fibroblast growth factor
G-CSF: granulocyte colony-stimulating factor
H&E: hematoxylin and eosin
IGF: insulin-like growth factor
IVF: in vitro fertilization
MSCs: mesenchymal stem cells
NS: normal saline
PDGF: platelet-derived growth factor
PRP: platelet-rich plasma
TGF-β: transforming growth factor beta
VEGF: vascular endothelial growth factor

Results

None of the dogs showed clinical complications associated with anesthesia, surgery, or intrauterine injection procedures. In the NS+PRP group, uterine tissue exhibited a normal structure, although mild hyperemia was observed in some sections, which was considered within normal physiological limits. The endometrial cylindrical epithelium remained intact and continuous, and the density of submucosal glands was preserved at normal levels. No pathological alterations, including hemorrhage, necrosis, or hemosiderosis were detected (Figure 1). In contrast, the E and E+NS groups showed severely reduced endometrial thickness due to extensive necrosis. Epithelial regeneration was poor and intermittent in some sections. In addition, glandular density was significantly diminished, with only minimal evidence of tissue regeneration. Submucosal cellularity was severely reduced, and significant hemosiderosis associated with extensive hemorrhage was observed in these tissues (Figure 2). Conversely, the E+PRP group showed substantial histological improvement following treatment. Endometrial thickness increased significantly accompanied by marked tissue repair. Re-epithelialization of the endometrium was highly successful, with approximately 90% epithelial healing observed. A considerable degree of uterine gland regeneration was also evident. Following tissue repair, submucosal cellularity increased, while pathological changes, including hyperemia, hemorrhage, and hemosiderosis, were markedly reduced (Figure 3).

As presented in Table 1, the E+PRP group demon-

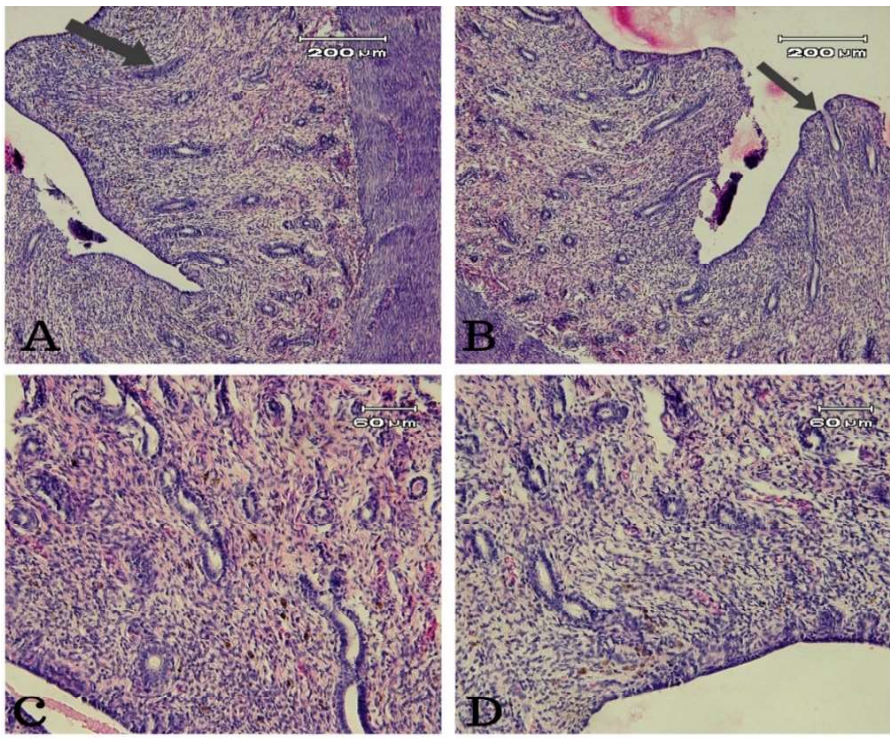

Figure 1.
Uterus of dogs, NS+PRP group.
The figure illustrates normal uterine tissue structure, featuring normal uterine glands (arrow in A) located in the endometrial submucosa, as well as integrated epithelial tissue (arrow in B) observed in all sections of the healthy group. In all pictures, the density of uterine glands and the cellularity of the submucosa were found to be consistent and normal. H&E staining.

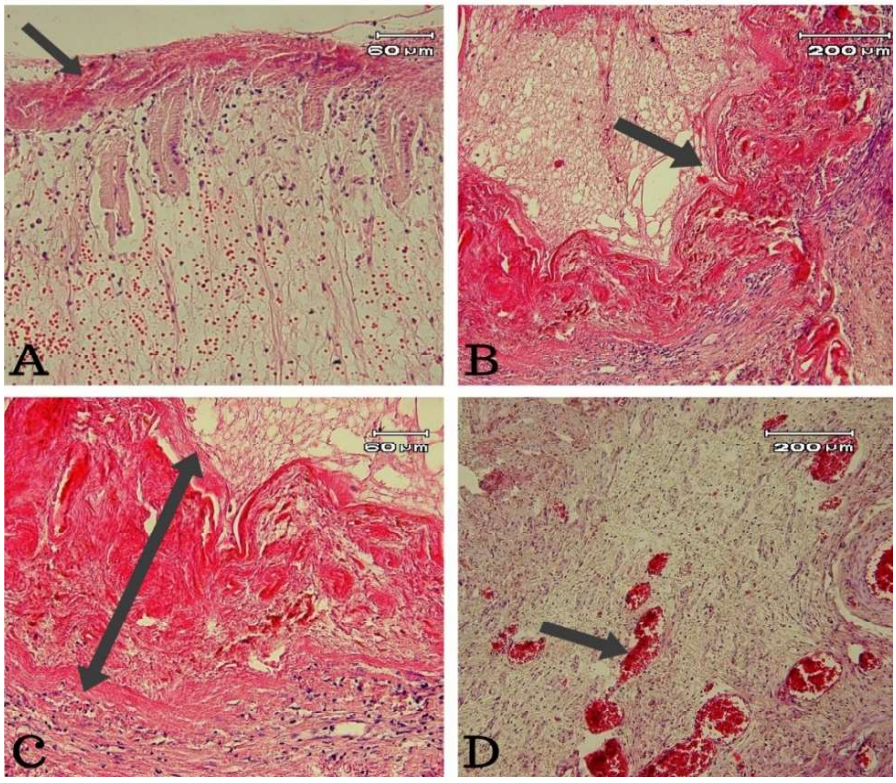

Figure 2.
Uterus of dogs, E and E+NS groups.
The figure shows extensive necrosis of the endometrial epithelium (arrows in A and B), accompanied by severe hemorrhage and hyperemia of the submucosal vessels (arrow in D). Additionally, there was a significant reduction in endometrial thickness due to necrosis and marked destruction (arrow in C) of the epithelium, submucosa, and uterine glands. H&E staining.

strated significant improvements in endometrial thickness, epithelial repair, and uterine gland density compared with the E group ($p < 0.05$). Conversely, the E+NS group showed no significant differences in these items compared with E group. The findings also indicated reduced severity of hemorrhage and necrosis in the E+PRP group compared with the E group, indicating a positive trend in improvement. In contrast, the E+NS group exhibited no changes or improvement trends relative to the E group. Furthermore, the levels of hyperemia and hemosiderosis in both the E+PRP and E+NS groups did not differ significantly from those in the E group (Table 1), though, E+PRP group showed relatively improved outcome (Figure 3).

Table 1.
Histopathologic findings in different experimental groups.

Parameter	Groups					
	E	E+NS	E+PRP	NS+PRP	Unit	P-values
Epithelial healing	10[a]	10[a]	90[b]	100[c]	%	0.025
Endometrial thickness (µm)	4.22 ± 0.91[a]	4.75 ± 1.73[a]	9.76 ± 3.41[b]	12.87 ± 4.67[c]	Mean ± SD	0.036
No. of uterus glands	3.19 ± 0.51[a]	4.22 ± 0.36[a]	21.50 ± 2.08[b]	37.62 ± 4.84[c]	Mean ± SD	< 0.0001
Hyperemia	2.0 (1.0-3.0)[a]	2.0 (1.0-2.0)[a]	1.0 (0.0-1.0)[a]	0.0 (0.0-0.0)[b]	Median (IQR)	0.025
Hemorrhage	3.0 (2.0-3.0)[a]	2.0 (2.0-3.0)[a]	1.0 (1.0-2.0)[b]	0.0 (0.0-0.0)[b]	Median (IQR)	0.036
Cellularity	2.0 (2.0-3.0)[a]	2.0 (1.0-2.0)[a]	1.0 (0.0-1.0)[b]	0.0 (0.0-1.0)[b]	Median (IQR)	0.052
Hemosiderosis	2.0 (1.0-2.0)[a]	2.0 (1.0-3.0)[a]	1.0 (0.0-1.0)a	0.0 (0.0-0.0)[b]	Median (IQR)	0.059
Necrosis	3.0 (2.0-3.0)[a]	2.0 (2.0-3.0)[a]	1.0 (1.0-2.0)[b]	0.0 (0.0-1.0)[b]	Median (IQR)	0.036

a,b,c: different letters in each row indicate statistically significant difference ($p < 0.05$)
NS: normal saline, E: ethanol, PRP: platelet rich plasma, IQR: interquartile ranges, SD: standard deviation.

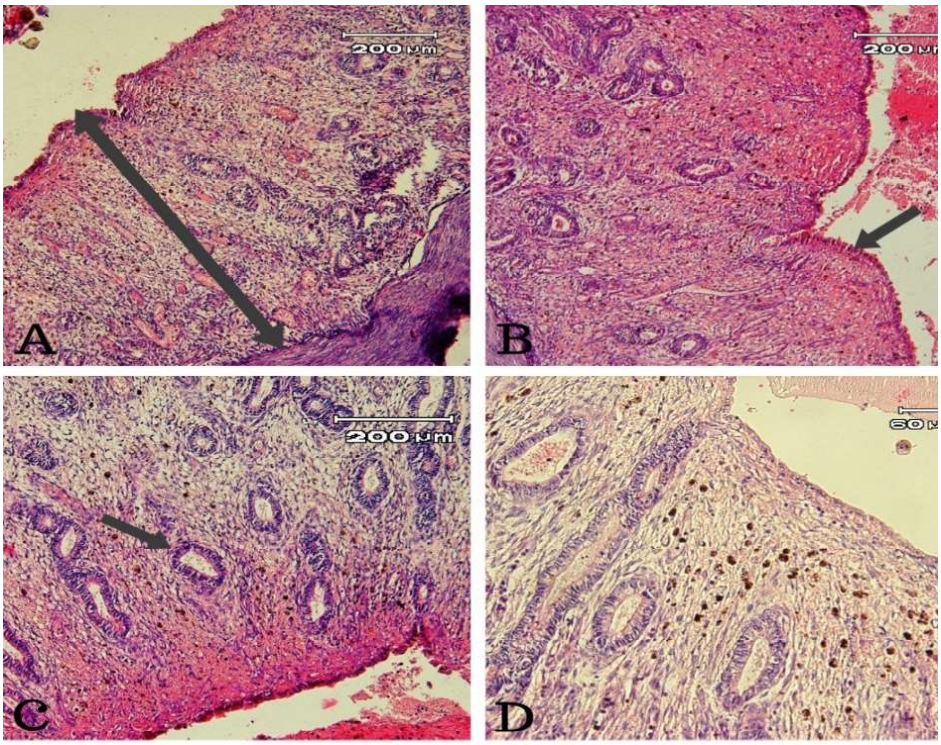

Figure 3.
Uterus of dogs, E+PRP group. This figure illustrates the improvement and repair of the submucosa (arrow in A) and increased cellularity, as well as significant healing of the endometrial epithelium (arrow in B) and regeneration of uterine glands (arrow in C). Additionally, the presence of hemosiderosis (brown cells in D) indicates prior bleeding and its subsequent healing. H&E staining.

Discussion

Endometrial thickness is a key factor in successful embryo implantation during in vitro fertilization procedures [17, 18]. Routine clinical causes of endometrial thinness include diminished production of VEGF and low levels of estrogen, leading to various proposed treatments; however, results have often been unsatisfactory [19]. Consequently, treating thin endometrium stays a significant problem in reproductive medicine.

In the present study, ethanol-induced endometrial atrophy in dogs was used as an experimental model to evaluate the beneficial impact of PRP on the thin endometrium. The results demonstrated that PRP increased the proliferation of endometrial mesenchymal and epithelial cells and facilitated blood vessel development, resulting in increased endometrial thickness and a more favorable environment for embryo implantation. Observations of uterine morphology

and histopathological examination further showed that the reparative effect of PRP extended beyond the change in endometrial thickness, as PRP treatment also improved uterus external morphology. PRP effectively improved uterine stricture and discoloration as a result of ethanol injury, returning the uterus to its normal condition. In the injured group, a decrease in the parameters including endometrial cells, glands, and blood vessels, along with epithelial cells loss, was observed. Conversely, in the group treated with PRP, these conditions improved, particularly in terms of glandular number and cell density. PRP may enhance endometrial receptivity by promoting cell proliferation, vascularization, and anti-inflammatory properties while reducing fibrosis, supported by concentrated peptides, growth factors, and cytokines, leading to improved endometrial thickness [8].

In all ethanol treated groups, hemosiderin deposits were observed within the endometrial tissue, indicating previous hemorrhage and erythrocyte degradation. Hemosiderosis is recognized as a histological marker of tissue injury and repair, reflecting phagocytosis of extravasated erythrocytes and iron accumulation within macrophages rather than ongoing necrosis [20, 21]. The presence of hemosiderin across groups, including those subsequently treated with PRP, indicates that vascular damage had occurred but was undergoing resolution, supporting the interpretation of healing rather than persistent injury.

Regenerative medicine, defined by Mason & Dunnill (2007), focuses on replacing or regenerating human tissue or organ cells to restore their normal function [22]. This innovative approach is particularly significant in treating chronic diseases that currently lack definitive cures. Key components of regenerative medicine include stem cells, growth factor-rich platelets, biological proteins, and gene therapy. The primary benefits of this treatment are its capability to regenerate tissue and the reduced risk of adverse reactions from allografts. Among the various cell types, platelet cells have garnered attention due to their rich growth factor content and ease of access [23]. Current research is exploring autologous blood products, such as PRP, to leverage the beneficial effects of growth factors in tissue repair. These growth factors release from alpha granules of the platelets, playing a fundamental role in the process of healing [24]. PRP acts as a source of chemical mediators during inflammation, releasing factors like VEGF, TGF, and EGF, which regulate migration of cells, adhesion, proliferation, and differentiation, while enhancing aggregation of the extracellular matrix [25, 26]. As was also shown in the present study; the utilization PRP has been shown to improve endometrial tissue in dogs. It is worth noting that its utilization has minimal risk of disease transmission or immune reactions due to its autologous preparation [27]. In this study, no infections or damage were observed following PRP injections.

The growth factors contained within PRP have established roles in angiogenesis, epithelial proliferation, and tissue repair. In canine PRP preparations, these mediators have been quantified at biologically active concentrations [28, 29], supporting their relevance in reproductive tissue remodeling. Mechanistically, VEGF promotes neovascularization within the endometrium, thereby improving blood supply, reducing hemorrhage and ischemic necrosis. PDGF stimulates stromal fibroblast and smooth muscle proliferation, facilitating extracellular matrix deposition and the expansion of endometrial thickness. In addition, TGF-β1 modulates epithelial stromal interactions and attenuates excessive inflammatory cellularity, contributing to a more organized tissue architecture. Collectively, these pathways provide a plausible explanation for the histological findings observed in this study, including increased endometrial thickness, improved glandular density, alongside reduced hemorrhage, cellular infiltration, and necrosis in the canine uterus under a thinned endometrium model. Although direct uterine studies in dogs are limited, the regenerative potential of PRP in canine tissues have been demonstrated in musculoskeletal and cutaneous healing contexts [30], and the same growth factor driven mechanisms can be reasonably extrapolated to endometrial repair. The present findings therefore align with the known biological activity of PRP growth factors in dogs and extend their application to reproductive physiology, highlighting PRP as a promising adjunct in managing endometrial insufficiency and related fertility disorders.

It is important to recognize that endometrial regeneration differs substantially between humans and dogs, reflecting divergent reproductive strategies. In women, the functional layer of the endometrium undergoes cyclic shedding during menstruation followed by rapid regeneration from basalis stem/progenitor cells, enabling scar free repair and receptivity for implantation [31]. In contrast, dogs are non menstruating species in which the endometrium undergoes hormonally driven remodeling characterized by prolonged proliferative and secretory phases, slow regression, and limited regenerative turnover. Canine reproductive pathology more commonly involves disorders such as cystic endometrial hyperplasia and pyometra rather than thin endometrium [20]. These species differences highlight why thin endometrium is a major clinical concern in human infertility compared to dogs. However, despite the paucity of similar studies on the canine endometrium, the results of the present study are comparable to those from human

studies. PRP administration resulted in significant rise in endometrial thickness and successful pregnancies in individuals with thinned endometrium [32, 33, 8], or during frozen embryo transfer cycles [34]. These findings align with the results of the current study.

Few studies have investigated the underlying mechanisms of PRP treatment. However, available evidence suggests that PRP may enhance endometrial receptivity by promoting cell proliferation, increasing vascularization, and exhibiting anti-inflammatory properties, while also reducing fibrosis. These effects are likely mediated by the concentrated peptides, growth factors, and cytokines present in PRP, which contribute to endometrial thickening [8]. A recent study by Montolío et al. (2025) analyzed growth factor concentrations in canine PRP preparations using canine-specific ELISA kits, and demonstrated significantly higher levels of TGF-β1 and PDGF-BB, with a strong positive correlation between platelet concentration and TGF-β1 levels. This suggests that the in house method effectively concentrates platelets and growth factors in products like PRP and platelet lysate, indicating that platelet concentration may predict specific growth factor levels [28]. Although fertility outcomes were not evaluated in the current study, the observed histomorphological improvements provide an important experimental basis for future in vivo fertility trials in dogs. Similarly, Puente et al. (2020) reported that PRP could regenerate endometrial tissue by enhancing vascularity and thickness, ultimately leading to successful pregnancies [35].

The regenerative effects observed in this study can also be compared with other biologic therapies investigated in canine regenerative medicine. Mesenchymal stem cells (MSCs) derived from adipose or endometrial tissues have demonstrated paracrine activity that promotes angiogenesis, modulates inflammation, and supports tissue repair. Canine endometrial MSCs, for example, have been characterized as multipotent and shown to exert cytoprotective effects through their secretome, suggesting potential utility in reproductive tract regeneration [36, 37]. Similarly, G CSF has been employed in canine models to mobilize progenitor cells and enhance hematopoietic recovery, with recombinant canine G CSF improving immune function and demonstrating systemic regenerative potential [38, 39]. Compared with these cell based or cytokine approaches, PRP offers a practical autologous source of growth factors such as PDGF, VEGF, and TGF-β1, which directly enhance vascular integrity, epithelial repair, and glandular regeneration. In the present study, PRP treatment reduced necrosis, improved endometrial thickness, and restored gland density, paralleling the beneficial outcomes reported with MSCs and G CSF, but with fewer technical challenges related to cell isolation, expansion, or recombinant cytokine administration. These findings support PRP as a feasible adjunctive therapy in canine uterine injury models.

Several limitations of the present study should be acknowledged. The relatively small sample size, selected in accordance with ethical principles aimed at minimizing animal use, limits the generalizability of the findings and supports classification of this work as a pilot study. Nevertheless, PRP with its reservoir of angiogenic and immunomodulatory growth factors, may offer novel adjunctive therapy for canine reproductive disorders. Although PRP has not yet been extensively investigated in conditions such as pyometra, postpartum subinvolution, or repeated breeding failure, its regenerative effects in other canine tissues [30] suggest potential benefits for restoring endometrial integrity and enhancing reproductive outcomes.

In summary, platelet-rich plasma was successfully utilized to treat ethanol-induced thin endometrium in dogs. Compared with untreated injured animals, platelet-rich plasma demonstrated the ability to improve thickness of the endometrium in dogs via rising the number of stromal glands and cells, diminution of fibrous areas, and elevating angiogenesis. In conclusion, this study offers both a theoretical and experimental foundation for the clinical application of PRP in treating thinned endometrium.

Materials and Methods

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this report, the authors used PopAi tool to rewrite some parts of the manuscript and check its grammar. After using this tool/service, the authors review and edit the content if needed and maintain full accountability and responsibility for the content of their work.

Ethical approval

All experimental procedures were performed in accordance with institutional and were approved by University/Regional Research Ethics Committee, University of Tabriz (approval ID: IR.TABRIZU.REC.1399.075).

Animals and study groups

In the present study, 12 healthy adult mixed breed dogs were included, with a mean body weight of 35 ± 4 kg and a mean age of 14 ± 2 months. The animals were maintained under controlled environmental conditions, at an average temperature of 25 °C and a 12-hour light/dark cycle. Commercial food and water were provided ad libitum throughout the study period. Only dogs in the anestrus phase of their sexual cycle were included in the study. Animals without vaginal discharge were selected for inclusion. Furthermore, those with small and smooth ovaries and quiescent uterus during laparotomy were entered to the study. To experimentally induce weak endometrium and endometrial damage, 95% ethanol was administered into the uterus, according to

previously described methods [40, 41]. The dogs were randomly assigned into four experimental groups: Group 1 (E) underwent 95% ethanol injection into uterine horns to induce endometrial damage; Group 2 (E+NS) received normal saline 72 hours after the uterine injury; Group 3 (E+PRP) received platelet-rich plasma 72 hours post-injury; and Group 4 (NS+PRP) received normal saline in both uterine horns and platelet-rich plasma 72 hours after initial injection.

Preparation of platelet-rich plasma

To prepare PRP, 20 ml blood was collected from the cephalic vein using a sodium citrate tube. The blood samples were subsequently underwent centrifugation at 1000 × g for 5 minutes, resulting in the formation of three distinct layers: platelets and white blood cells formed the upper layer, the buffy coat which is full of white blood cells was the middle layer, and the red blood cells formed the lower layer. The upper and middle layers were carefully transferred into a sterile tube to obtain leukocyte rich PRP. The separated plasma underwent a second centrifugation at 1500× g for 15 minutes using a double-spin protocol. Following centrifugation, the upper two-thirds of the platelet-poor plasma was discarded, leaving 3 ml above the pellet. The remaining pellets, which represent the platelet-rich plasma, were then homogenized and resuspended in the residual plasma to obtain about 5.3× the baseline concentration [42, 43].

Preoperative preparation and exploratory laparotomy

Exploratory laparoscopy was utilized for administration of ethanol, normal saline, and platelet-rich plasma into the uterine horns. Prior to the procedure, premedication was administered with 0.05 mg/kg of 1% acepromazine (IM, Alfasan, Worden, The Netherlands). General anesthesia was induced using 5 mg/kg of 10% ketamine (IM, Alfasan, Worden, The Netherlands) and 0.25 mg/kg of 0.5% diazepam (IV, Kimia Daru, Iran). Following endotracheal intubation, anesthesia was maintained with halothane in oxygen (Halothane BP, Nicholas Piramal India limited, India). During surgery, intravenous serum therapy was administered using 0.9% normal saline (Sodium Chloride, I.P.P.C., Tehran, Iran) together with 1 g cefazolin (Cefazolin-Exir, Exir Co., Tehran, Iran). The surgical site was prepared aseptically, and an incision was made through the linea alba to access the abdominal cavity. After identification of the uterine horns, 3 to 5 ml of either non-toxic 95% ethanol (Groups E, E+NS; and E+PRP) or normal saline (Group NS+PRP) was injected into each horn. A second Laparotomy was performed 72 hours later as previously described, and 0.5 to 1 ml of normal saline (Group E+NS) or PRP (Groups E+PRP and NS+PRP) was injected into uterine horns, except for group E in which ovariohysterectomy was carried out at this second surgery for tissue collection . The abdominal wall was then sutured in three layers. Following the intrauterine injections and surgery, the dogs were monitored for their general condition and appetite.

In the experimental groups, ovariohysterectomy was conducted to collect uterine horn samples. Premedication and general anesthesia were administered as in previous surgeries. After incising the linea alba and locating the uterine horns, the ovarian pedicles were transected using the two-forceps technique. The broad ligament and arteries were ligated and cut. A sample was then taken from the bifurcation of the uterus after the uterine body was transected using transfixation and simple ligatures. An incision was made at the bifurcation and the sample placed in 10% formalin. The abdominal wall was sutured in three layers. The sutures were removed two weeks after surgery, during which the dogs were monitored for their general condition and appetite.

Tissue sampling and histopathologic studies

In group E, tissue sampling was performed 72 hours after the injection of 95% ethanol. For the E+NS, E+PRP, and NS+PRP groups, which assessed the effects of normal saline and platelet-rich plasma in uteri with damaged endometrium, or PRP effects in normal endometrium, ovariohysterectomy was performed 14 days after the final intrauterine injections. Collected uterine tissues were transferred to the pathology laboratory for sampling, sectioning, and slide preparation. Samples were fixed in 10% buffered formalin at a volume at least ten times that of the samples. After 24 hours, the formalin was replaced, and sections were prepared from the uterine tissue for processing. The tissue preparation steps included dehydration using ascending alcohol concentrations, clarification with xylene, and removal of alcohol, followed by embedding in molten paraffin. Sections of 4 to 5 µm in thickness were prepared using a microtome, placed in an oven at 56 °C for thirty minutes, and then stained with hematoxylin and eosin. The prepared slides were evaluated by a pathologist unaware of the groups, using a conventional light microscope (Olympus-CH30, Japan) for endometrial thickness, epithelial healing, and uterine gland density. Other potential pathologic lesions, including necrosis, hyperemia, hemorrhage, cellularity, and hemosiderosis, were also examined. The mean of 3 measurements per uterine horn were calculated to account for heterogeneity. In cases where no lesions were observed, the normal state was assigned a grade of 0, mild lesions got a grade of 1, moderate lesions were given a grade of 2, and severe lesions obtained a grade of 3.

Statistical analysis

Quantitative data were analyzed using ANOVA, while the observed histopathological findings were considered semi-quantitative data, analyzed through non-parametric methods including Kruskal-Wallis for between-group comparisons, followed by post-hoc Dunn's tests for pairwise differences. SPSS statistical software (SPSS for Windows, Version 22, Inc., Chicago, Illinois, USA) was utilized to analyse the data, with a confidence level set at 95%. A P-value of less than 0.05 was accepted as statistically significant .

Authors' Contributions

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

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Competing Interests

The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

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Preparation and in vitro evaluation of canine hyperimmune plasma against *canine parvovirus*: a strategy for passive immunotherapy

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ABSTRACT

The aim of this study was to prepare anti-canine parvovirus hyperimmune plasma through repeated vaccination of dogs and to evaluate its neutralizing capacity and immunoreactivity against viral antigens in comparison with plasma from healthy and infected animals. Four healthy dogs were vaccinated four times at two-week intervals using a live attenuated CPV-2 vaccine and hyperimmune plasma was collected. A CPV-2b strain was isolated from fecal samples of clinically infected dogs and propagated in MDCK cells. Viral identity was confirmed by PCR targeting the VP2 gene. Neutralizing antibody titers were determined using a virus neutralization assay, while CPV-specific antibody titers were measured by indirect ELISA. The immunogenicity against viral structural and non-structural proteins was assessed using Western blot analysis. Hyperimmune plasma exhibited the highest neutralizing titer (1:160±64) compared with healthy (1:26 ± 12) and infected (1:4.5 ± 2.5) groups ($p \leq 0.01$). ELISA optical density values were 0.63 ± 0.11, 0.50 ± 0.12, and 0.28±0.02 for hyperimmune, healthy, and infected plasma, respectively ($p \leq 0.001$). Western blot analysis revealed the presence of reactive antibodies against VP2 (66.0 ± 7.2), VP1 (30.7 ± 14.3), VP3 (45.0 ± 5.3), and NS1 (68.0 ± 7.1) in the hyperimmune group, whereas only low levels of antibodies against VP2 were detected in infected dogs. While antibodies against NS1 were observed exclusively in the hyperimmune plasma. In conclusion, Canine hyperimmune plasma contains high-titer, polyclonal neutralizing antibodies against CPV. These in vitro findings support the probable effectiveness and its potential utility as an emergency passive immunotherapy for CPV infection.

Keywords

Canine parvovirus, Hyperimmune plasma, Passive immunotherapy, Dog

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Number of Tables: 3
Number of References:: 27
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Abbreviations

CPV: Canine parvovirus
HIP:Hyperimmune plasma
MDCK: Madin-Darby Canine Kidney cell line
PCR: Polymerase Chain Reaction

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Introduction

Canine parvovirus Type 2 (CPV-2) is a highly pathogenic viral agent responsible for hemorrhagic gastroenteritis and acute leukopenia in young dogs, often leading to high mortality rates if untreated [1]. This non-enveloped, single-stranded DNA virus belongs to the Parvoviridae family, subfamily Parvovirinae, and is characterized by rapid genetic evolution [2]. Since its initial identification in the late 1970s, three major antigenic variants, CPV-2a, CPV-2b, and CPV-2c, have been identified and have circulated worldwide, with CPV-2c being the predominant type in many regions [3]. In Iran, recent studies indicate that all three variants are present, with CPV-2a remaining the predominant type [4, 5, 6]. Clinical signs of the disease include severe vomiting, hemorrhagic diarrhea, dehydration, leukopenia, and fever. In untreated cases, mortality rates may reach up to 90% [7]. Currently, no definitive treatment exists; clinical management relies on supportive therapy, including intravenous fluids, electrolyte replacement, broad-spectrum antibiotics, antiemetics, and pain control [8].

Nevertheless, these methods impose high costs on pet owners and often lack sufficient therapeutic efficacy [9]. In recent years, passive immunotherapy has gained attention as a complementary treatment approach. This method involves the administration of pre-formed antibodies derived from external sources, such as hyperimmune sera or concentrated immunoglobulins, into the sick animal to directly neutralize the virus and prevent disease progression [10]. Numerous studies have reported positive effects of passive immunization, including reduced hospitalization duration, decreased viral load, and improved survival rates [11, 12]. However, immunological reactions following serum and antibody injections from different animal species, the unavailability of hyperimmune serum for native strains, the high cost of imported products, and the risk of microbial contamination remain significant challenges in many regions, including Iran [13].

Repeated vaccination of healthy animals with live attenuated virus can lead to the production of hyperimmune sera containing high titers of neutralizing antibodies [14]. These sera can serve as a source for producing therapeutic or diagnostic products. In a study by Kotb et al. (2015), hyperimmune serum produced in horses was able to completely prevent mortality caused by CPV, when administered within the first 72 hours post-infection [10]. Given the conflicting results of previous studies on the efficacy of hyperimmune serum and the need to develop high-quality domestic sources, the present study was designed to prepare and immunologically evaluate canine hyperimmune plasma against CPV-2 using in vitro assays. Active immunity was induced in healthy dogs, and the titers of neutralizing antibodies were assessed through virus neutralization, ELISA, and Western blot assays. Additionally, the antibody response patterns against the viral structural proteins (VP1, VP2, and VP3) and the non-structural protein (NS1) were compared to explore the potential use of this serum in therapeutic and diagnostic applications.

Abbreviations-Cont'd

VP1, VP2, and VP3: viral structural proteins
NS1: Non-structural protein

Results

Assessment of initial health and immune response to vaccination

All four dogs underwent a two weeks quarantine period and screening tests, during which fecal tests for parasites and CPV shedding tests returned negative results. Following completion of the quarantine phase, all animals proceeded to the immunization phase. After administration of four doses of the live attenuated vaccine, no local or systemic adverse effects were observed. The mean serum volume collected from each dog was 260 ± 20 ml. No microbial contamination or hemolysis was detected in any of the samples.

Virus isolation, confirmation and genotyping of the isolated virus

Based on the results, cytopathic effects were observed in the cell culture (Figure 1A). Molecular confirmation demonstrated successful amplification of the expected gene fragments, approximately 583, 420 and 682 bp in length, in positive samples. agarose gel electrophoresis (1.5%) revealed distinct bands at expected position in positive lanes, while no band was observed in the negative control (Figure 1B). These findings confirm that the isolated sample contained the Canine parvovirus genome. Additionally, PCR-based genotyping results showed that the isolated virus yielded positive amplification with the CPV-2b/2a and CPV-2b specific primers. These PCR genotyping sets indicate that the isolated viruses belongs to the CPV-2b strain.

Neutralizing antibody titer in plasma

The mean neutralizing titer in hyperimmune sera (160 ± 64) was significantly higher than that observed in the healthy group (26 ± 12) and the infected group

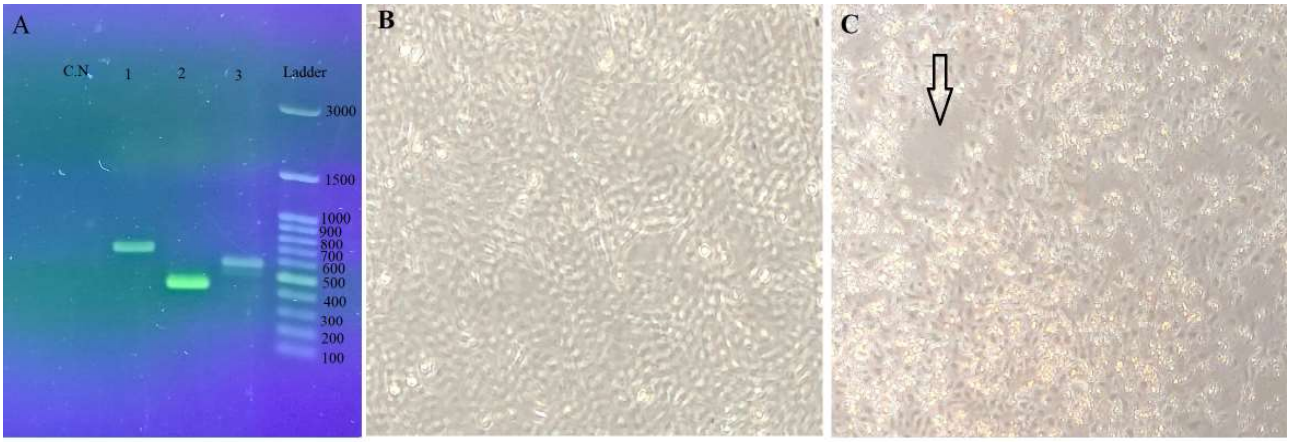

Figure 1.
Confirmation and genotyping of Canine parvovirus extracted from faecal samples of infected dogs. A) Confirmation and genotyping of isolated Canine parvovirus strain. A) Agarose gel electrophoresis of PCR products for the detection of VP2: (1) the PCR product of "Pab" primer pairs, (2) the PCR product of "Pb" primer pairs and (3) the PCR product of "555forc and 555revc" primer pairs. B) Normal culture of Madin-Darby Canine Kidney (MDCK) cell line. C) Occurrence of cytopathic effect (CPE) on the cell culture (indicated by arrow).

(4.5 ± 2.5) ($p \leq 0.001$). The highest individual titer (1:256) was observed in one dog within the hyperimmune group (Table 1). The Spearman correlation between the neutralizing titer and ELISA optical density was r = 0.89 ($p \leq 0.001$).

Specific anti-CPV antibody titer

The mean optical density (OD) value in hyperimmune group was 0.63±0.11, in the healthy group 0.59 ± 0.12, in the infected group 0.28 ± 0.02, and in the negative control sample 0.14 ± 0.004. Pairwise comparisons showed that both hyperimmune and healthy groups had significantly higher antibody levels than the infected group ($p \leq 0.001$) and the control group ($p \leq 0.001$). Additionally, the infected group showed significantly higher values than the control group ($p \leq 0.001$). However, no significant difference was observed between hyperimmune and the healthy group ($p > 0.05$)

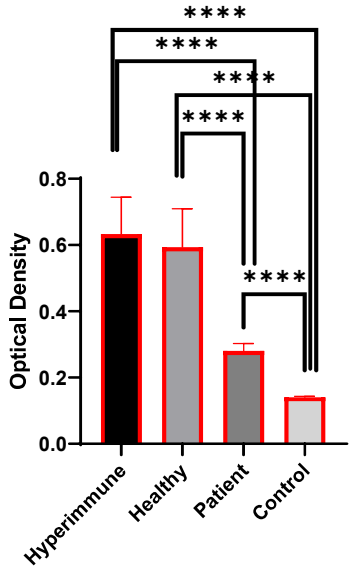

Figure 2.
The specific anti-Canine parvovirus titer in healthy, infected, and hyperimmune dogs evaluated by ELISA. The significant difference greater than 0.001 showed by four stars

Table 1.
Neutralizing titer of parvovirus by plasma from healthy, infected, and hyperimmune dogs.

Dog IDs	SN titer for Hyperimmune Dogs	SN titer for Healthy Dogs	SN titer for infected Dogs
1	1:128	1:32	1:8
2	1:256	1:32	1:2
3	1:128	1:8	1:4
4	1:128	1:32	1:4
Overall Mean	1:160 ± 64	1:26 ± 12	1:4.5 ± 2.5

Western blot results

The intensity of the VP2 band in hyperimmune group was 66.0 ± 7.2 luminous units, which was 1.6 times higher than the healthy group and 50 times higher the infected group (Table 2 and Figure 3A and 3B). VP1 and VP3 proteins were clearly detected in hyperimmune and healthy sera, with intensities of 30.7 ± 14.3 and 45.0 ± 5.3 units, respectively. The NS1 was specifically present in hyperimmune sera (68.0 ± 7.1 units), healthy (12.9±1.5 and remained below the detection threshold in infected group (≤ 2 units). Dot blot analysis of whole virus particles yielded results

Table 2.
Average intensity of Western blot bands in plasma analysis of healthy, infected, and hyperimmune dogs with Canine parvovirus (optical units ± standard deviation). ND: below the detection threshold. Statistically significant differences are indicated by different lowercase letters.

Antigen	Hyperimmune	Healthy	Infected	Statics
Whole-Virus	170.2 ± 9.5 [a]	91.2 ± 15.0 [b]	2.7 ± 2.0 c	F=312, $P \leq 0.001$
Vp1	30.7 ± 14.3 [a]	6.1 ± 2.9 [b]	ND	t= 4.8, $P = 0.039$
Vp2	66.0 ± 7.2 [a]	41.6 ± 3.4 [b]	1.3 ± 0.9 c	F=189, $P \leq 0.001$
Vp3	45.0 ± 5.3 [a]	11.9 ± 0.3 [b]	ND	t=18.7, $P = 0.003$
NS1 (N)	68.0 ± 7.1 [a]	12.9 ± 1.5 [b]	2.1 ± 1.8 c	F=215, $P \leq 0.001$

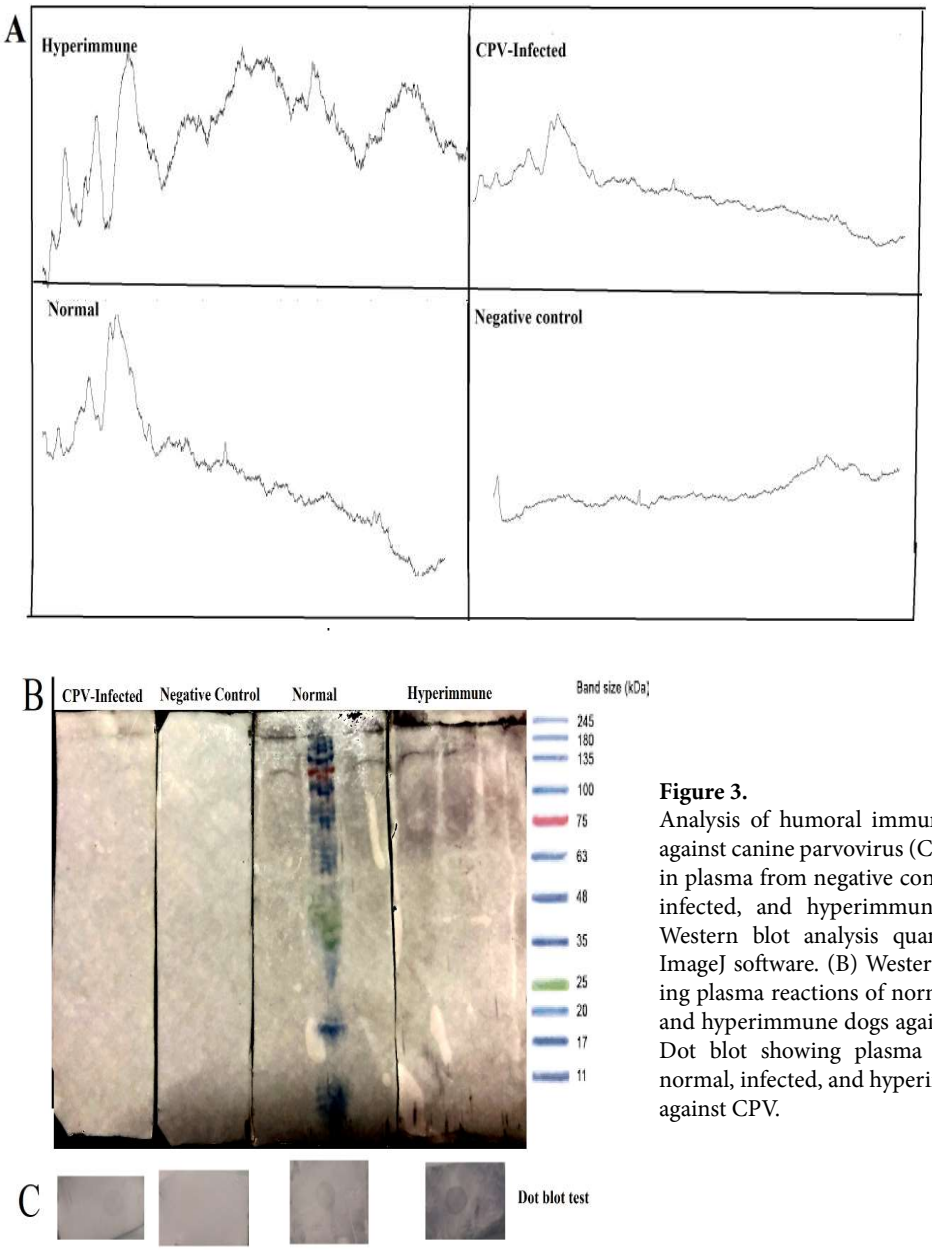

Figure 3.
Analysis of humoral immune responses against canine parvovirus (CPV) antigens in plasma from negative control, normal, infected, and hyperimmune dogs. (A) Western blot analysis quantified using ImageJ software. (B) Western blot showing plasma reactions of normal, infected, and hyperimmune dogs against CPV. (C) Dot blot showing plasma reactions of normal, infected, and hyperimmune dogs against CPV.

consistent with western blot assay, showing that the dot corresponding to hyperimmune plasma exhibited the highest reaction intensity, while the infected plasma showed the lowest intensity (Figure 3C).

Discussion

The aim of this study was to prepare and conduct in vitro evaluation of canine hyperimmune plasma against canine parvovirus. In the present study, canine

parvovirus was propagated in the MDCK cell line, with cytopathic effects (CPE) first observed on day three post infection. Although A-72 and CRFK/F81 cell lines are frequently employed for Canine parvovirus propagation, previous reports have demonstrated that MDCK cells are also permissive to CPV infection and capable of supporting productive viral replication [15]. Nevertheless, replication efficiency and absolute infectivity titers may vary among different cell lines. In the present study, all virus titration and neutralization assays were performed using the same cell line and virus stock, ensuring valid relative comparisons of neutralizing antibody responses between experimental groups. Additionally, PCR-based genotyping demonstrated that the isolated CPV strain belong to the CPV-2b variant. The virus neutralization assay provides a functional assessment of antibody-mediated inhibition of viral infectivity, supporting the relevance of these findings for passive immunotherapy. While $TCID_{50}$-based virus neutralization assay in current study provides a functional evaluation of antibody-mediated inhibition of viral infectivity; the inclusion of molecular quantification and standardized neutralization metrics could further enhance analytical resolution.

The appearance of cytopathic effects (CPE) at day three suggests a high replication capacity of the local CPV strain in MDCK cells. This is consistent with Kaur et al [15], who reported CPE appearance on the fifth day. The shorter time to CPE appearance in the present study may be attributed to two factors: the use of freshly passaged cells (less than 24 hours old), which are more sensitive to the virus, as well as the presence of mutations in the GH loop (positions 297 and 426) that enhance the virus's binding to the TLR receptor [16]. These findings indicate that the optimal culture conditions (37 °C, 5% CO_2, and DMEM supplemented with 2% FBS) are sufficient for the rapid replication of the studied CPV strains.

The mean titer of 1:160 in hyperimmune sera was significantly higher than that of normal serum (1:26) and infected sera (1:4.5) ($p \leq 0.001$). This 50-fold difference is consistent with two key studies; Kotb et al. (2015) reported a titer of 1:024 in horses (10), and Larson et al. (2024) recorded a titer of 1,320 in dogs receiving monoclonal antibodies, resulting in a 100% reduction in mortality [12]. Similarly, Dash et al. (2020), reported 25% reduction in mortality rate following administration of hyperimmune serum [11]. The neutralization assay using CPV-2b strain and antibody titer showed significant difference among evaluated sera. The result could be interoperated as necessity of enhancement of humoral immunity of the infected animals in acute phase of the disease using hyperimmune sera or polyclonal IgG. The hyperimmune serum used in this study was prepared using CPV-2 vaccine, which is designed to provide protection against all CPV-2 strains. Therefore, the resulting hyperimmune serum may be effective in infected animals against various variants of virus.

The detection of VP2, VP1, VP3, and NS1 exclusively in hyperimmune sera, along with the absence of VP1 and VP3 in the patient's serum, aligns with the findings of Nelson et al. (2007) [14]. They reported that these proteins are expressed at low levels (<5%) in parvoviruses and elicit a weak immune response during natural infection. Furthermore, NS1, detected clearly only in hyperimmune sera. This observation is consistent with the study by Miao et al. (2022), who demonstrated that antibodies against NS1 appear only after repeated vaccination [17]. Based on current result, the infected animals in acute phase of the disease, have significant reduced titer against CPV-2 in all in-vitro tests. So, the lower immunogenic protein like as NS1 was appeared only in hyperimmune animals or healthy animals. These proteins may therefore have potential to be used for serologically diagnosis of the animals in acute phase of the disease.

Spearman's correlation coefficient was 0.89 between neutralization titer and ELISA titer, and 0.85 between ELISA optical density and the sum of Western blot band intensities, indicating that these three methods are highly interchangeable. This finding is consistent with Decaro et al. (2006), who reported a coefficient of 0.82 between the neutralization and ELISA tests [18]. Therefore, in clinical settings where neutralization tests are time consuming, ELISA with a cutoff OD ≥ 0.45 (sensitivity 92% and specificity 88%) may serve as a rapid screening tool.

Asil et al. (2023), reported that CPV-2 disrupts B cell differentiation and suppresses both humoral and cellular immune responses by significantly reducing IL-6, CD4+, and CD8+ levels in peripheral leukocytes. This condition is associated with a severe form of the disease in dogs (CPVD-Severe), characterized by leukopenia, thymic atrophy, and high mortality. The reduction in antibody titers in affected dogs compared to healthy dogs can be attributed to this immune suppression, as well as to the neutralization of antibodies by the virus during the acute phase of the disease [19]. Similarly, Ulas et al. (2024), indicated that using standard supportive treatment, the survival of the infected animals was only 57.1%, whereas the addition of recombinant interferon-ω improved the neutrophil/lymphocyte ratio and increased circulating lymphocytes, raising survival to 85.7% [20]. These finding indicates that cellular immune recovery during the acute phase of the disease is critical, as

supportive therapy alone may be insufficient in severe symptomatic cases .

Given the suppression of active immunity during the acute phase of the disease, passive immunotherapy represents as the most rational therapeutic strategy. In the current study, the hyperimmune serum exhibited a neutralization titer of 1:160 and an OD_{450} of 0.63, demonstrating 50 times higher titer than the neutralizing capacity of infected serum. In addition to targeting VP2, the hyperimmune serum also produced antibodies against VP1, VP3, and NS1, providing multi-epitope coverage and reducing the risk of antigenic escape. According to a simplified pharmacokinetic model, canine IgG has a half-life of 8–10 days. Following intravenous injection of 5–10 mg/kg of hyperimmune serum with a titer of 1:160, serum levels of ≥ 1:80, which considered as a protective titer [21, 22], may be maintained for more than 72 hours. This period coincides with peak viremia (days 3 to 5 post infection), providing sufficient opportunity for initial control of infection before severe occurrence of intestinal damage. In the present study, the neutralizing titer of the prepared hyperimmune serum was 1:160, which is lower than that of commonly imported products (typically 1:512); while simultaneously decreasing dependence on imports conditions. However, considering the lower cost of domestic production, it can reduce treatment expenses by up to 50%. Therefore, despite lower absolute titers, the locally produced hyperimmune serum is not only pharmacokinetically effective but also may represent as a more economically viable and accessible therapeutic alternatives.

Conclusion

For the first time in southwestern Iran, a CPV-2b variant was isolated and characterized, demonstrating high replication capacity in MDCK cells. Multi-steps vaccination of healthy dogs with a live attenuated vaccine induced the production of indigenous hyperimmune sera with a neutralization titer of 1:160, significantly higher than that observed in both healthy and naturally infected dogs. Western blot analysis identified VP2 as the dominant immunogenetic antigen, while VP1, VP3, and especially NS1 were detected in hyperimmune sera and healthy dogs. The method described in current study for preparation of canine hyperimmune serum using CPV-2 vaccination yielded promising in vitro results. The prepared hyperimmune serum or its purified polyclonal IgG can be manipulated for preparation of a safe and endotoxin free product that could be receive license for in vivo evaluation of its ppharmacokinetics and efficacy in infected dogs with parvovirus.

Materials and Methods

Vaccination and preparation of hyperimmune sera

All experimental procedures, including animal care, treatment, and sample collection, were conducted in accordance with the ethical guidelines approved by the Ethics Committee and the regulations for working with animals at Shahid Chamran University of Ahvaz, under approval number 1404.088. In this study, four young adult female indigenous dogs, aged between 1.5 and 2.5 years, were obtained and individually housed in separate cages for two weeks. During this period, clinical examinations were performed, and a complete anthelmintic drug (Caniverm) was administered. After confirming the animals' health and the absence of prior parvovirus infection using an immunochromatography test kit (Hangzhou Evegen Biotech, China), a specific monovalent parvovirus vaccine (BioVeta brand) was administered subcutaneously in four doses at two-week intervals. Ten days after the final injection, a total of 500 mL of blood was collected from each dog in two occasions at two weeks intervals using special blood transfusion bags. Plasma was separated using standard centrifugation methods and stored at –20°C until efficacy testing was performed.

Collection of fecal and blood samples

Four dogs presenting at the Veterinary Faculty Clinic of Shahid Chamran University of Ahvaz tested positive for parvovirus antigen via immunochromatography kit. From each case, 5 g of fecal sample and 2 mL of blood were collected and transported to the Virology laboratory. Fecal samples were homogenized in sterile PBS and centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and filtered through 0.22 µm syringe filters for decontamination. The filtered liquid was then treated with antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL) prior to virus isolation. Plasma was separated from the collected blood samples using standard methods. Additionally, plasma samples from four healthy dogs, which tested negative for the antigen and showed no clinical signs, were also prepared.

Virus isolation

Virus isolation was performed using the MDCK (Madin-Darby Canine Kidney) cell line cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal bovine serum (FBS). MDCK cells were used for virus isolation as a canine origin epithelial cell line that has previously been reported to support the replication of canine parvovirus with typical cytopathic effects [15]. When cell monolayer reached approximately 40–50% confluence, the cells were infected with 0.5 mL of virus-containing fluid and incubated at 37°C for 1 hour to allow virus adsorption. Following adsorption, inocula were removed, cells were washed with sterile PBS, an appropriate volume of DMEM containing 2% FBS was added to the flasks, which were then incubated at 37°C in a 5% CO2 atmosphere for 3-4 days until cytopathic effects (CPE) were observed. Following the appearance of CPE, the flasks were stored at -20°C, then thawed. The contents were transferred to sterile Falcon tubes and cell debris was removed by centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was used as the virus stock [13]. The virus infectivity (TCID50) of the stock was determined using the Reed-Muench method to ensure the appropriate virus concentration for the virus neutralization assay [16].

PCR test for confirmation and genotyping of the isolated virus

To confirm that observed CPE resulted from Canine parvovirus infection and not nonspecific cytotoxicity, the identity of the propagated virus was molecularly confirmed by PCR amplification of a VP2 gene fragment (583 bp) from infected cell cultures [23]. Furthermore, to determine the CPV genotype of the isolated virus, two separate PCR assays were performed using genotype-specific primer sets targeting CPV-2a/2b, and CPV-2b, respectively [24], as detailed in Table 3. Nucleic acids were first extracted from the viral source using the Sinapure DNA extraction kit (Sinaclon Company, Iran) following the manufacturer's instructions. PCR was then performed in a 25 µL reaction volume, comprising 12.5 µL of Amplicon 2x master mix, 1 µL each of forward and reverse primers at 10 µM concentration, 7.5 µL of nuclease-free water (Sinaclon Company, Iran), and 3 µL of template DNA. The cycling profile for all primer sets and rounds included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes. PCR products were electrophoresed alongside a 100 bp DNA ladder (Sinaclon, Iran) on a 1.5% agarose gel containing safe dye (Sinagen, Iran) at 100 volts for 60 minutes and visualized under UV light using a transilluminator (Uvtec, England).

Table 3.
Sequence of the used primers for identification and genotyping of *Canine parvovirus*.

Primer name	Sequence (5′→3′)	Amplicon size (bp)	Target Genotype	Reference
555forc	CAGGAAGATATCCAGAAGGA	583	All	[23]
555revc	GGTGCTAGTTGATATGTAATAAACA			[24]
Pbs	CTTTAACCTTCCTGTAACAG	428	B	[24]
Pbas	CATAGTTAAATTGGTTATCTAC			
Pabs	GAAGAGTGGTTGTAAATAATT	682	A / B	
Pabas	CCTATATAACCAAAGTTAGTAC			

Virus neutralization test

The infectivity of the prepared virus stock was calculated as the 50 % tissue culture infectious dose (TCID50) using the Reed and Muench method [16]. Subsequently, a dose of 100 TCID50 was used in the virus neutralization assay. The neutralizing capacity of hyperimmune plasma was compared with plasma of healthy and infected dogs with Canine parvovirus. After collecting plasma samples from each group, the samples were filtered through 0.22 µm syringe filters for potential decontamination. To remove non-specific and inhibitory factors present, the plasma samples were first incubated at 55°C for 30 minutes. Each sample was then diluted at a ratio of 1:2. Next, 100 TCID50 of the active virus was mixed with an equal volume of the diluted plasma. To facilitate the binding reaction between the antibodies (present in the plasma) and the antigen (virus), the mixture was incubated at room temperature for 1 hour. Subsequently, MDCK cells were co-cultured in 96-well plates with each dog plasma dilution mixed with the virus, in triplicate. The microplates were incubated at 37°C in 2% CO2 and monitored daily under a microscope. At the end of the incubation period, the neutralizing antibody titer against parvovirus in the tested plasma samples was determined based on the presence or absence of cytopathic effects.

Assessment of parvovirus-specific antibody titer

Inactivated and freeze-thawed antigens of isolated parvovirus strain from infected dogs were diluted in carbonate-bicarbonate buffer (pH 9.6) and coated onto ELISA plates (Jet Biofil, China) at a concentration of 1 µg/well. The plates were incubated at 4 °C overnight. After that they been washed with PBS containing 0.05% Tween 20 (PBS-T). The plates were blocked by addition 250 µL of 4% skimmed milk to each well and incubation for 2 hours at room temperature. After additional three washing step same as before, plasma samples from 4 healthy dogs, 4 infected dogs, and 4 hyperimmune dogs, diluted according to optimized recipe, were added to each well and incubated for 1 hour at room temperature. Following another washing step, rabbit anti-dog IgG at 10 µg/mL was added, followed by another one-hour incubation at room temperature. Addition 100 µL of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Immuno Chemistry Technologies Company, USA) at a 1:20,000 dilution and incubation at room temperature for one hours was conducted in followed step. In the final step, 60 µL of tetramethylbenzidine (TMB) substrate was added to each well, and after 10 minutes, 60 µL of stop solution was added. The optical density of each sample was measured at 450 nm using a spectrophotometer.

Antibody reaction with parvovirus immunogenic proteins

A Western blot test was conducted to compare the pattern of immunogenic proteins of parvovirus in infected, hyperimmune, and healthy dogs. Freeze-thawed viral antigens at equal concentrations equal to 200 µg/mL were used for this assay. Initially, antigens were mixed with sample buffer containing 0.1 M beta-mercaptoethanol and boiled for 10 minutes. A total of 25 µL was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for 120 minutes. The antigens were then transferred to nitrocellulose membranes using the wet transfer method. Active sites on the membranes were blocked with 4% skimmed milk overnight at 4°C. Optimized 5% serum dilutions from infected, hyperimmune, and healthy dogs were separately applied to each sample lane. Subsequently, a secondary Anti-dog antibody of rabbit origin was added at a concentration of 10 µg/mL, followed by an HRP-conjugated antibody diluted 1:7.500. In the final step, alpha-chloronaphthol substrate was added, and after 15 minutes

of incubation in the dark, the membranes were washed with distilled water to stop the reaction. Photographs of the test results were taken, and the molecular weight and intensity of the viral immunogenic protein bands in the test groups was defined using ImageJ software. The molecular weights of the Western blot bands were used to identify the corresponding CPV antigens based on previous studies. The visible bands with molecular weights of 82.3, 64, 65.7, and 76.6 kDa corresponded to VP1, VP2, VP3, and NS1, respectively [25-27]. Dot blot assay was performed by coating 1 µL of CPV antigens (200 µg/mL) onto PVDF membrane. The membrane was incubated at room temperature until dry. Afterward, the membranes were washed three times with PBS-T buffer and blocked with a 4% skim milk solution for 2 hours. The subsequent steps were carried out as described in the Western blot section.

Statistical analysis

The study results were analyzed using SPSS software with a one-way analysis of variance (ANOVA) method. Statistical significance was set at $p \leq 0.05$.

Authors' Contributions

All authors read and approved the final manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Effects of intracerebroventricular injection of nisin on feeding behavior, body temperature, and serum parameters in LPS-inflamed broiler chickens

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ABSTRACT

Lipopolysaccharide (LPS) leads to impaired production performance, reduced feed intake, altered body temperature, and deleterious changes in some serum and disease parameters in broiler chickens. Bacteriocins such as nisin are considered promising therapeutic agents for the prevention, control, and treatment of poultry diseases. This study involved an experiment using 30 male broiler chickens to evaluate the central effects of nisin on feed and water consumption, body temperature, and serum factors under stress. In this experiment, nisin (1 µg) and LPS (25 ng) were injected intraventricularly, and combined treatments of nisin and LPS were also considered. Feed intake, water consumption, and body temperature were measured in all groups. At the end of the experiment, blood was collected from the chickens for evaluation of serum factors. ICV administration of nisin administration significantly increased feed intake, whereas LPS administration significantly decreased it. In combination treatments, nisin was able to partially reduce the adverse effects of LPS on feed intake. Both nisin and LPS reduced water intake in broilers, and combined treatment caused a greater reduction in water intake than either injection alone. LPS initially reduced body temperature before hyperthermia induction, whereas nisin alone had no effect on thermoregulation, but in the combination groups, it somewhat reduced LPS-induced temperature disturbances. LPS also caused a significant decrease in albumin and a significant increase in AST and ALT, whereas nisin reduced AST and ALT but had no effect on albumin. Results shows that nisin can be used as a useful and safe additive in poultry feed.

Keywords

Nisin, LPS, Feeding behavior, Temperature, Serum parameters, Broilers

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Abbreviations

ICV: intracerebroventricular
IV: intravenous
LPS: lipopolysaccharide
ARC: arcuate nucleus

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Introduction

Feed consumption in poultry is regulated by a multifaceted network of central and peripheral control systems driven by hormonal, metabolic, neural, and environmental factors [1]. Among the central regulatory structures, the hypothalamus is the endocrine host of feeding behavior and energy homeostasis [2]. Various hypothalamic regions, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventromedial hypothalamus (VMH) and dorsomedial nucleus (DMN) are known contributors of this regulatory mechanism [3]. These nuclei respond to circulating hormones and metabolites and subsequently regulate feeding behavior and body temperature [4].

In intensive poultry-rearing systems, broilers are continuously expose to environmental stressors and pathogenic microorganisms, that can suppress immune system activities and reduce productive performance [5]. Lipopolysaccharide (LPS) is an important component in the cell wall structure of Gram-negative [6]. Following entry of LPS into the broiler chicken's body, the animal undergoes an extensive acute phase response featuring decreased feed intake and impaired growth, inflammation with associated changes to its thermoregulation [7]. Its weight loss component is not only reflected by suppressed appetite, but also by metabolic/endocrine modification induced by inflammatory cytokines [8].

The high-LPS-rich outer membrane of Gram-negative bacteria serves as an essential protective barrier and plays a critical role in host–pathogen interactions and antibiotics susceptibility [9]. The structure of LPS consists of lipid A, core oligosaccharides, and O-antigens that determines membrane permeation and resistance patterns determines membrane permeation and resistance patterns[10]. It imparts innate resistance to the majority of antibiotics and antimicrobial peptides and thus, is a key contributor towards the increasing worldwide prevalence of antimicrobial resistance (AMR) [11].

Gram-negative organisms are especially worrisome, with resistance to even the most recent compounds described [12]. As a result, there is increasing interest in the discovery of new antimicrobial agents acting specifically against pathogenic bacteria with minimal impact on the commensal microbiota [13].

In this background, bacteriocins have gained prominence as potential candidates. Such peptides usually have a relatively narrow antibacterial spectrum, low cytotoxicity, and high potential for treating MDR bacteria [14].

Nisin is a class Ia lantibiotic produced by Lactococcus lactis and has been extensively studied for its broad-based antimicrobial efficacy and safe nature [15]. Nisin is approved by the U.S. Food and Drug Administration (FDA) as a natural food preservative and is widely recognized for its effectiveness against various Gram-positive pathogens. Although traditionally considered ineffective against gram-negative bacteria, accumulating evidence suggests that nisin may also exhibit activity against Gram-negative bacteria [16].

Mechanistic data suggest that nisin is able to interact with LPS and disturb the outer membrane, most likely in strains with truncated LPS cores then enabling lipid II access or channel formation [17]. Moreover, findings from solid-state nuclear magnetic resonance (NMR) analyses and leakage assays have demonstrated that LPS chemical structure plays a significant role in the sensitivity of bacteria to nisin detecting those with rough forms is stronger [18].

Apart from its antimicrobial properties, nisin has a high immunomodulatory and anti-inflammatory effect [19]. Nisin was observed to demonstrate a reduction in excessive cytokine production and the regulation of immune cell signaling pathways [20]. These properties may exemplify its ability to sustain physiological homeostasis within the stressed animals and to improve resilience against immunologically challenging pathological agents [21]. In poultry, the immunomodulatory role of nisin have been associated with improvements in feed intake and growth performance. Evidence suggests that nisin improves growth performance, relives inflammation-related anorexia, regulates heat production, and alleviates dissipation in broiler chickens challenged immunologically [22].

Previous studies have established that dietary nisin supplementation improves growth performance and feed intake in the early rearing phase of broiler

Abbreviations-Cont'd

PVN: paraventricular nucleus
LHA: lateral hypothalamic area
VMH: ventromedial hypothalamus
DMN: dorsomedial nucleus
AMR: antimicrobial resistance
MDR: multidrug-resistant
NMR: nuclear magnetic resonance
HPA: hypothalamic–pituitary–adrenal
BBB: blood–brain barrier
FCR: Feed Conversion Ratio
TNF-α: tumor necrosis factor-alpha
IL-1β:interleukin-1 beta
IL: interleukin
NPY: neuropeptide Y
AgRP: agouti-related peptide
POMC: pro-opiomelanocortin
CART: cocaine- and amphetamine-regulated transcript
FDA: Food and Drug Administration

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chickens [23]. However, despite substantial evidence supporting its antimicrobial and immunological activities, the central mechanisms through which nisin influences feeding behavior, especially under conditions of LPS-induced inflammatory stress, are still inadequately known. Moreover, the effects of nisin on body temperature regulation, energy balance, and serum biochemical parameters have not been clearly elucidated.

Consequently, the present study was conducted to investigate the central effects of nisin on feeding behavior, water intake, body temperature, and serum parameters in lipopolysaccharide-challenged broiler chickens. More specifically, we examined intracerebroventricularly infused nisin, which may affect feed intake, rescue the LPS-dependent decrease in feed consumption, alter temperature and serum parameters, and lower the negative LPS effect. This research was also conducted to further elucidate the potential for nisin to serve as a functional feed additive for broiler chickens.

Results

Base on the results, ICV administration of an effective dose of nisin significantly increased cumulative feed intake compared with the control group at 90, 120, and 180 minutes post injection ($p \leq 0.05$). In contrast, ICV administration of an effective dose of LPS significantly decreased cumulative feed intake at 30, 60, 90, 120, and 180 minutes post injection compared with the control group ($p \leq 0.05$). Co-administration of nisin and LPS, significantly improved the LPS-induced reduction in feed intake, especially at 90, 120, and 180 minutes post injection ($p \leq 0.05$) (Figure 1).

Effects of ICV administration of nisin significantly reduced water intake at 30, 60, 90, 120, and 180 minutes post injection ($p \leq 0.05$); ICV administration of LPS also significantly reduced water intake at 30, 60, 90, 120, and 180 minutes post injection ($p \leq 0.05$). Co-administration of nisin and LPS, exhibited a greater reduction in water intake compared to the other groups ($p \leq 0.05$) (Figure 2).

As concerns body temperatures, no significant differences were observed between the nisin-treated and control groups ($p > 0.05$). In contrast, following LPS injection, body temperatures initially decreased and subsequently increased above the normal values. Co-administration of nisin with LPS significantly attenuated the LPS-induced hyperthermia ($p \leq 0.05$) (table 1).

The effects of the treatments on serum parameters revealed that there were no significant differences in the nisin-injected groups compared with the control group ($p > 0.05$). However, in the LPS-treated groups, ALB concentrations were significantly lower than those of the control group ($p \leq 0.05$). A similar reduction in ALB levels was also observed in the nisin and LPS co-treated groups ($p \leq 0.05$), illustrating the inability of nisin to inhibit the reduction of ALB caused by LPS. Conversely, the value of both AST and ALT were significantly higher in the LPS-treated groups compared with the control groups ($p \leq 0.05$), whereas in the nisin and LPS co-treated groups, nisin was able to reduce the impact of LPS on the mentioned serum parameters ($p \leq 0.05$) (table 2).

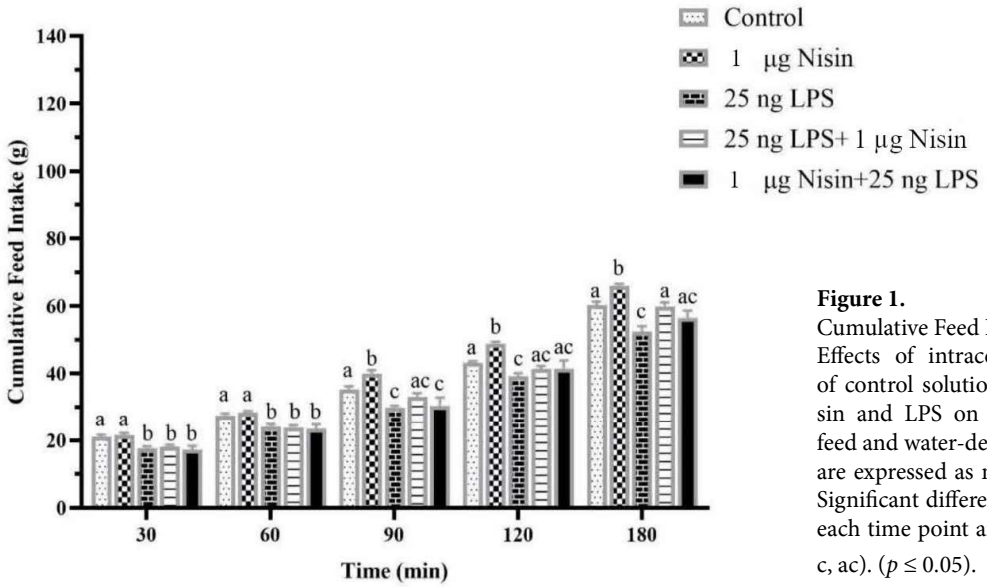

Figure 1.
Cumulative Feed Intake
Effects of intracerebroventricular injection of control solution and effective dose of nisin and LPS on cumulative feed intake in feed and water-deprived broilers (N=6). Data are expressed as mean ± standard deviation. Significant differences between treatments at each time point are indicated by letters (a, b, c, ac). ($p \leq 0.05$).

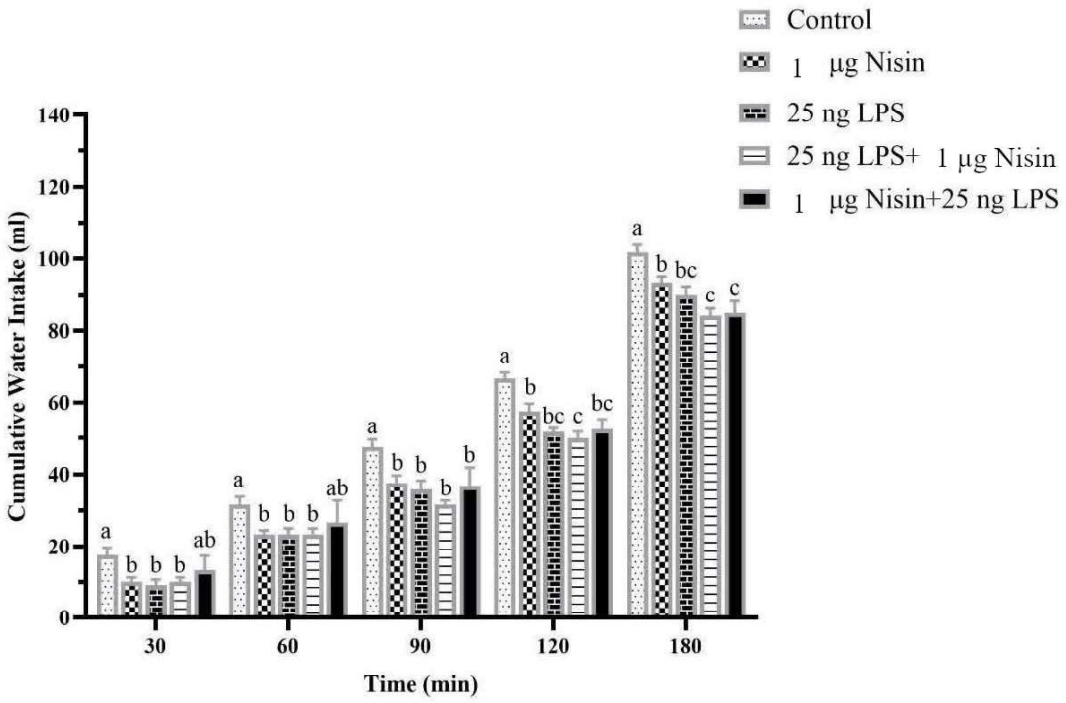

Figure 2.
Cumulative Water Intake
Effects of intracerebroventricular injection of control solution and effective dose of nisin and LPS on cumulative water intake in feed and water-deprived broilers (N=6). Data are expressed as mean ± standard deviation. Significant differences between treatments at each time point are indicated by letters (a, b, ab, bc, c). ($p \leq 0.05$).

Table 1.
Effects of nisin and LPS on body Temperature in the experiment.

Groups	10 min before inj.	1 h after inj.	3.5 h after inj.	5 h after inj.	8 h after inj.
1	40.76 ± 0.07^{a}	40.83 ± 0.04^{a}	40.78 ± 0.10^{a}	40.83 ± 0.06^{a}	40.83 ± 0.09^{a}
2	40.75 ± 0.05^{a}	40.76 ± 0.05^{a}	40.75 ± 0.08^{a}	40.83 ± 0.09^{a}	40.73 ± 0.06^{a}
3	40.86 ± 0.14^{a}	40.05 ± 0.07^{b}	40.88 ± 0.08^{a}	41.25 ± 0.09^{b}	42.11 ± 0.08^{b}
4	40.85 ± 0.11^{a}	39.86 ± 0.10^{b}	40.83 ± 0.07^{a}	40.95 ± 0.08^{ab}	41.16 ± 0.03^{c}
5	40.78 ± 0.06^{a}	39.76 ± 0.09^{b}	40.68 ± 0.11^{a}	40.85 ± 0.09^{a}	40.93 ± 0.06^{ac}

Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time ($p \leq 0.05$).

Discussion

Nisin is a polypeptide bacteriocin produced by Lactococcus species, capable of improving broiler growth performance while exhibiting antioxidant and anti-inflammatory properties, which has led to growing interest in its application [24]. LPS, a component of Gram-negative bacteria, is commonly used to induce systemic inflammation in experimental animals. Previous studies have shown that LPS challenge significantly reduces weight gain, feed intake, and feed conversion ratio [25]. The present study is investigating the effects of ICV administration of nisin on feed intake in LPS-challenged broilers. Compared with the extensive research on nisin activity against Gram-positive bacteria, relatively few studies have addressed its effects on Gram-negative bacteria and their associated inflammation.

The results demonstrated that ICV administration of nisin increased feed intake at 90, 120, and 180 minutes post-injection. These finding are consistent with previous studies demonstrating that dietary supplementation with nisin enhances growth performance

Table 2.
Effects of nisin on some serum biochemical parameters of LPS-stimulated broilers

Groups	Cre	GLU	TG	Urea	BUN	ALP	ALB	AST	ALT
Control	0.23 ± 0.02	271.00 ± 1.71	77.00 ± 2.44	5.83 ± 0.16	2.67 ± 0.21	2763.00 ± 225.99	1.78 ± 0.04^{a}	274.33 ± 7.46^{a}	8.33 ± 0.42^{a}
Nisin	0.28 ± 0.01	266.33 ± 4.45	75.00 ± 2.17	5.17 ± 0.30	2.33 ± 0.21	2815.00 ± 175.95	1.78 ± 0.04^{a}	262.83 ± 4.50^{a}	8.17 ± 0.60^{a}
LPS	0.25 ± 0.02	255.67 ± 3.94	76.33 ± 3.45	5.00 ± 0.36	2.33 ± 0.21	2793.67 ± 241.46	1.35 ± 0.04^{b}	296.33 ± 2.40^{b}	10.17 ± 0.30^{b}
LPS + Nisin	0.27 ± 0.02	265.67 ± 7.81	76.83 ± 3.57	5.00 ± 0.25	2.33 ± 0.21	2712.33 ± 231.66	1.40 ± 0.03^{b}	278.83 ± 3.19^{ab}	9.83 ± 0.30^{ab}
Nisin + LPS	0.25 ± 0.02	264.33 ± 8.07	76.17 ± 3.68	4.83 ± 0.30	2.50 ± 0.22	2705.83 ± 231.24	1.42 ± 0.06^{b}	276.50 ± 2.26^{a}	9.50 ± 0.22^{ab}

Data are expressed as mean ± SEM. Abbreviations: Cre: Creatinine, GLU: Glucose, TG: Triglycerides, Urea: Urea, BUN: Blood Urea Nitrogen, ALB: Albumin, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase. Different letters (a, b and c) indicate significant differences between each treatment in the respective factors ($p \leq 0.05$).

and feed intake in broiler chickens, including Ross 308 strains [26]. In group 5 of the experiment, nisin was administered first, followed by LPS injection, to evaluate the potential preventive effects of nisin.

In the present study, it can be observed that the initial administration of nisin was effective in reducing the adverse effects of LPS, as it partially reduced the effects of LPS on food and water intake, temperature, and even some serum parameters. This can be related to earlier studies showing that nisin suppresses the Production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [27], while promoting the production of regulatory mediators such as IL-10 and TGF-β [28].

In another study, nisin supplementation increased intestinal villus height, which may further contribute to improved broiler growth performance [23].

In another study, nisin supplementation increased intestinal villus height, which may further contribute to improved broiler growth performance [24]. In the present study, ICV administration of LPS reduced feed intake, consistent with previous studies .The faster and stronger effects observed via the ICV route may be explained by bypassing the blood–brain barrier (BBB) and directly influencing hypothalamic nuclei and neuropeptides related to hunger and satiety, such as the arcuate nucleus (ARC) and paraventricular nucleus (PVN), through modulation of neuropeptides including NPY, AgRP, POMC, and CART [29].

LPS has a damaging effect on intestines and their barrier, which could be a reason for lowered growth rates in LPS-inoculated broilers. Previous studies have shown that LPS affects the gene and protein structure of tight junctions in the intestines, thereby compromising nutrient absorption and water-electrolytes secretion [30]. The pro-inflammatory cytokine TNF-α, produced as a result of exposure to LPS, further contribute to the deterioration of tight junction proteins [31].

In broiler chickens, LPS-triggered anorexia has been mediated by decreased expression of orexigenic neuropeptides, such as Neuropeptide Y (NPY) and Agouti-Related Peptide (AgRP) in the hypothalamus, as well as activation of the Hypothalamus-Pituitary-Adrenal (HPA) axis, along with changes in nutrient partitioning from growth to immune processes, such as antibody production and inflammatory cytokines [32].

In our experiment, the co-administration of LPS and nisin suggested that nisin has the potential to counteract the adverse effects of LPS on feed intake. The previous study has already validated that nisin has anti-inflammatory activity and nisin supplementation can suppress the Production of TNF-α and IL-6, while simultaneously promote the Production of IL-2, indi-

cating the stimulation of innate immunity [27].

In this study, besides the effect of nisin on cumulative feed intake, water intake was also measured. By comparison with energy balance, water balance or the regulation of body fluids for homeostasis is more complex as it includes a variety of biological functions such as the cellular and molecular regulation of water intake and excretion [33]. Various organs can also be involved in osmoregulation and fluid regulation. In birds, these organs include the kidney, intestine, and salt gland [34].

In the peresent study, ICV injections of nisin were associated with a significant reduction in water intake. The relationship between feed and water intake in the LPS-injected group was linear; however, this pattern was not observed in the nisin-treated group. The inverse association between feed and water intake, which is less common in poultry, may be influenced by several factors, including breed, environmental temperature, sex, growth rate, diet composition, and physicochemical properties of feed ingredients [35]. Additionally, the stimulation of specific nuclei in the central nervous system could potentially promote appetite and, at the same time, limit water consumption [36].

The challenge, illustrated by the conflicting effects on feed and water intake, indicates that nisin potentially impacts water intake in a unique way via the central nervous system. The impact of nisin on water intake in poultry has not been documented earlier, and in a study on nisin intake in mice, the findings illustrated an increase in water intake [37].

The paraventricular nucleus, supraoptic nucleus, and medial-basal hypothalamus are involved in the production and stimulation of neuropeptide Y, which occurs during osmotic stress and dehydration and these nuclei likely contribute to water regulation in chicks [38]. Because the paraventricular nucleus receives signals related to blood volume status, it may involve in the inhibition of NPY production in nucleus, and therefore it can play a role in stimulating drinking behavior [39].

Although both nisin and LPS caused reduced water intake, and co-administered nisin and LPS caused further reduced water intake as opposed to controls, the processes and physiological effects of nisin and LPS might not be the same.

Also, intestinal health is significant for water and electrolyte uptake in poultry and is very important for water balance [40]. Intestinal disorders or inflammation can result in impaired water uptake, causing diarrhea, thereby increasing water consumption and enteric infections also facilitate water excretion in the feces, apart from causing disturbances in electrolyte balance[24]. Nisin, with the capacity to reduce inflammation of the epithelial lining, causing a modification in the intestinal microbiota, can be very effective in the uptake of sodium, thus potentially reducing water consumption[27].

Overhydration in broilers may lead to increased moisture and saturated bedding that makes the broiler flocks prone to diseases such as ascites and contact dermatitis [41]. Adequate regulation of water consumption is also important to lower the chances of developing physiological ailments such as tibial dyschondroplasia, ascites, cardiovascular diseases, and

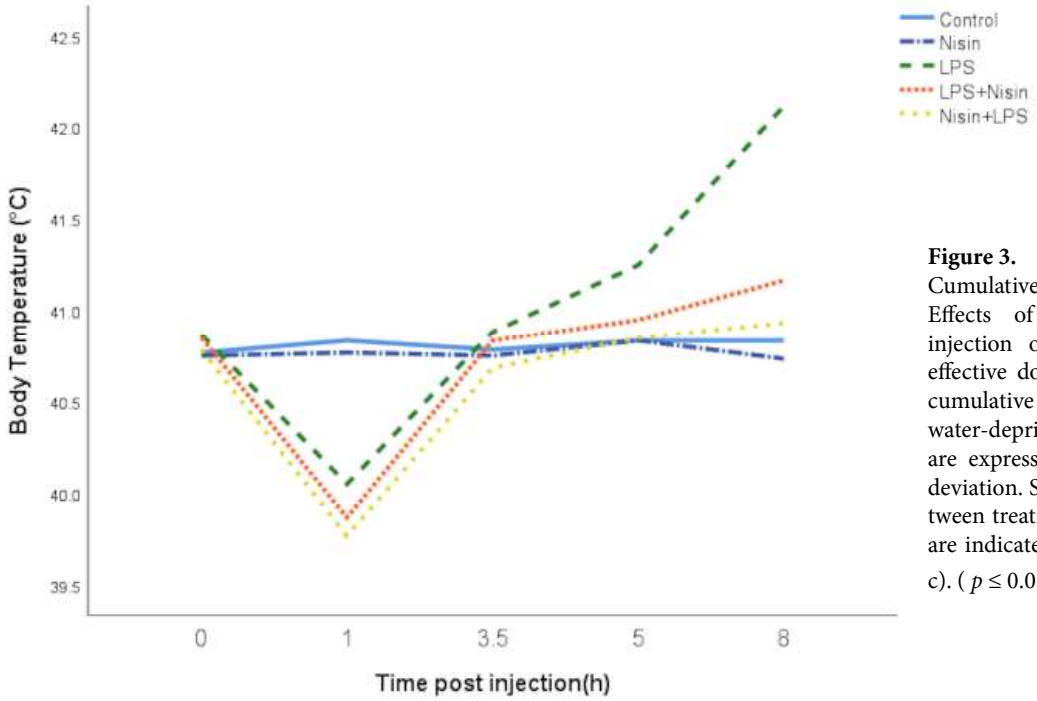

Figure 3.
Cumulative Water Intake
Effects of intracerebroventricular injection of control solution and effective dose of nisin and LPS on cumulative water intake in feed and water-deprived broilers (N=6). Data are expressed as mean ± standard deviation. Significant differences between treatments at each time point are indicated by letters (a, b, ab, bc, c). ($p \leq 0.05$).

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reproductive failure[42]. In our study, cloacal temperature measurements 10 minutes before the start of the experiment and 1, 3.5, 5, and 8 hours after injection confirmed the effects of LPS. Showing an initial drop followed by increased temperature in LPS-treated groups. Similar findings have been reported in earlier studies, where LPS IV-injection elevated cloacal temperature three hours post-administration [43]. Another study also observed increased body temperature and reduced appetite in LPS-challenged broilers and reported that strong LPS doses initially decreased then increased body temperature in chickens [44]. Notably, in the present study, nisin was able to somewhat reduce the adverse effects of LPS on body temperature among the intervention groups. Figure 3 shows the temperature changes in each experimental group during the experiment, and in Table 1, the groups are compared at different times, and the numbers are written as mean ± SEM.

The results of serum biochemical parameters, did not show significant differences between the nisin-treated and control group. In another study that investigated the effects of nisin on the blood serum of broiler chickens, no significant changes were observed in serum parameters except glucose [26]. The differences in the effects of the variables related to glucose may be attributed to different factors such as the duration of the experiment, the differences in the dose, and the experimental conditions. In another experiment conducted on rats, it was found that the consumption of nisin did not affect the liver enzymes AST and ALT, triglycerides, cholesterol, HDL, and LDL, which demonstrates that nisin is not harmful to the physiological health of the human body[45]. In a study on rabbits, nisin administered in drinking water did not lead to significant differences in total protein, triglycerides, cholesterol, glucose, calcium or ALT[46]. In the present study, the results of serum biochemical parameters in the LPS group showed an increase in the levels of ALT and AST enzymes. The activities of AST and ALT enzymes in serum are usually used as specific indicators of liver damage. The results of other studies showed that intraperitoneal injection of LPS increased the levels of ALT and AST [47]. Notably. in the present study, albumin levels decreased, and reduction in albumin concentration in the LPS group may also be attributed to liver injury.

The immune response in broiler chickens caused by LPS negatively affects their growth; this occurs mainly because of protein metabolism failure in the body. The results in the intervention groups show that nisin can significantly reduce the adverse effects of LPS on ALT and AST factors, but has no effect on the adverse effects of LPS on ALB factor.

Conclusions

This study shows that central injection of nisin can increase feed intake and reduce water consumption in broiler chickens, and reduces the negative effects of LPS on feed intake, body temperature, and some serum parameters. These findings provide new evidence for the potential role of bacteriocins, particularly nisin, in modulating feed and water intake and reducing the deleterious effects on body physiology in LPS-induced broiler chickens.

Materials and Methods

Ethical Considerations

The present study was conducted in accordance with ethical principles, national regulations, and standards for medical research in Iran.

Ethical approval ID: IR.UM.REC.1403.313, dated January 25, 2025.

All experiments were executed according to the Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal ethics committee.

Animals

A total of 30 one-day-old male broiler chicks of the Ross 308 breed were obtained from Fariman Company (Mashhad, Iran). During the first five days post-hatch, birds were maintained under normal rearing conditions. Broilers were housed in a controlled environment with temperatures of 30 ± 2° C for the initial 48-hour period, followed by a gradual reduction of approximately 2.5° C per week. Relative humidity was maintained at 50 ± 2%.

The lighting cycle adhered to conventional management practices, consisting of 23 hours light and 1 hour darkness during the first week, and gradually adjusted to a 18 hours light and 6 hour darkness photoperiod by the end of the experiment period. The broilers had free access to clean water and a commercial broiler starter feed (Sāleh Co., Kashmar) throughout the experiment.

The diet was formulated with a concentration of 23% crude protein, 2% crude fiber, 1% calcium, 0.5% available phosphorus, 0.55% methionine, 1% lysine, 1% methionine + cystine, 0.18% and 2900 kcal/kg metabolizable energy.

After the 5-day acclimation time, chicks were randomly allocated to five experimental groups (n = 6 per group). Prior to intracerebroventricular (ICV) injections, birds were fasted for 3 hours to minimize variation in metabolic status, while water remained available ad libitum.

Experimental Compounds

Nisin (Product Code: N5764-1G) was purchased from Sigma-Aldrich, USA. This bioactive compound, which belongs to Lactococcus lactis, has been defined to consist of 2.5% purified nisin along with sodium chloride and denatured milk derived components. This bioactive compound, manufactured and sold by Sigma-Aldrich, has been registered and indexed using CAS number 1414-45-5, and it possesses an empirical formula composed of $C_{143}H_{230}N_{42}O_{37}$. Lipopolysaccharide (LPS) was obtained from E. coli O111:B4 (Life Biolab, Cat. No. LB22429, Lot No. 20L9147).

All solutions were prepared using sterile, pyrogen-free physiological saline. A stock solution with 1 mg/ml nisin was prepared

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and subsequently diluted to obtain the desired experimental concentration (1 µg). A stock solution with 10 mg/ml LPS was prepared and subsequently diluted to obtain the desired experimental concentration.

Experimental Groups
Group 1: Control
Group 2: Nisin
Group 3: LPS
Group 4: LPS + Nisin
Group 5: Nisin + LPS

The time interval between the two injections of nisin and LPS in the fourth and fifth groups was 15 minutes.

Surgical Preparation

Stereotactic surgery was performed when the chicks were 18 days old and weighed approximately 750g. Food was withdrawn for one hour before surgery, while water w remained ad libitum. Anesthesia was induced via a face mask using isoflurane (5% isoflurane in 1 L/min oxygen) and maintained at 2–3% isoflurane (Piramal Critical Care Inc., Guayama, USA) in 1 L/min oxygen delivered through a tracheal tube. All broilers were confirmed to be clinically healthy following routine physical examination.

A 23-gauge thin-walled stainless steel guide cannula was stereotaxically implanted into the right lateral ventricle according to previously established coordinates: 6.7 mm anterior to bregma, 0.7 mm lateral to the midline, and 3.5-4 mm below the dura mater [48]. Finally, three stainless steel screws were inserted around the entry point of the cannula into the skull, and the cannula and the three screws were fixed in place with acrylic dental cement (Acropars, Tehran, Iran). In the control group, an orthodontic #014 wire was inserted into the guide cannula to maintain equivalent conditions.

To prevent postoperative infection, Linkospectin (Razak, Tehran, Iran) was applied topically to the incision site and administered intraperitoneally. Birds were allowed a minimum recovery period of five days, before the initiation of the subsequent injections.

Experimental Procedures

A single experiment was conducted to evaluate the effects of nisin on feed intake, water intake, body temperature, and serum biochemical parameters in broilers challenged with LPS-induced inflammation (n = 30). Each experiment consisted of one control group and four treatment groups. Body weight (BW) was consistent across all chickens (950 ± 50 g).

The injections were administered using a thin-walled stainless steel injection cannula of 30-gauge, which projected 1.0 mm beyond the tip of the guide cannula. The injection cannula was connected to a 10-µl Hamilton syringe with a 30-cm PE-20 polyethylene tube. The Lipopolysaccharide was given an infusion time of 60 seconds, and the control solution was infused into the lateral ventricle simultaneously. All tubes and syringes were stored in a 70% ethanol. Glassware was sterilized by an autoclave to maintain a Pyrogen-free environment. Sterilized physiological saline was used as a control solution.

Correct placement of the guide cannula within the right lateral ventricle was confirmed by the appearance of cerebrospinal fluid (CSF) and by ICV infusion of methylene blue, followed by methylene blue in the right place confirmation in frozen brain sections at the conclusion of each experiment. Only data from accurately performed injections were included in the statistical analyses.

Feed was withheld 3 hours prior to the start of the experiment. The chicken has been immediately returned into the cage after injection. The cumulative feed consumption (g) and water consumption (g) of the chicken were measured at 30 min, 60 min, 90 min, 120 min, and 180 min post-injection. At the time of experimentation, chicks were 23 days old.

Nisin and LPS were administered via the ICV route alone or in combination to assess the central nisin administration on LPS-induced inflammation. Chickens received 1 µg/kg of nisin dissolved in 10 µl saline. LPS was administered at a dose of 25 ng in 10 µl saline.

Because no prior studies have reported ICV administration of nisin in broilers, the selected dose was based on data from mammalian studies and preliminary pilot experiments conducted by the current research team. In contrast, the effective ICV dose of LPS in broilers (25 ng) has been previously established by some of the members of the present research team [49]. At the end of the experiments, blood samples were collected from the metatarsal vein.

Statistical Analysis

The cumulative amount of feed and water intake data was analyzed employing one-way repeated measures ANOVA in GraphPad Prism software, version 9.4.1 (GraphPad Software, USA). In order to determine statistical significance, the value of $p \leq 0.05$ was taken into consideration. After observing significant differences in treatment, the Tukey post-test comparison was conducted.

Variations that might exist between the body temperatures of the LPS-treated and control broilers and the serum biochemical values were estimated using the one-way ANOVA method. Statistical significance was accepted at $p \leq 0.05$.

Authors' Contributions

All authors contributed to the study conception and design. Vahid Hamedianasl performed material preparation, data collection, and investigation. Farshid Hamidi carried out project administration, supervision, methodology, conceptualization, review and editing of the manuscript. Hamidreza Kazerani carried out the formal analysis, software processing, validation, and visualization. Vahid Hamedianasl wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

Nisin, LPS, Feeding behavior, Temperature, Serum parameters, Broilers

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Morphological and molecular analyses of two Taenia species in wild canids in northern Iran

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ABSTRACT

Wild canids play a significant role in dissemination of parasitic helminth ova and may serve as a potential source of infection in humans and animals. Therefore, monitoring the helminth fauna of these species is essential for both veterinary and public health. The present cross-sectional study was conducted to assess cestode infection and to reveal the molecular characterization and phylogenetic status of large Taenia in wild canids in Iran. Descriptive statistics (frequencies and percentages) were used to summarize the data, and the Chi-square test was employed to evaluate differences in parasite distribution among different variables. For this aim, the intestines of 93 wild canids, including 69 jackals, 22 red foxes, and two wolves, were examined in northern, northeastern, and northwestern regions of Iran for tapeworms. Additionally, the identity of some helminths was confirmed using PCR and DNA sequencing. Based on our findings, 54.5% of red foxes and 47.8% (95% CI: 36.5-59.3%) of jackals were infected, among which single infections with *Mesocestoides* were highly prevalent (33.3% , 95% CI: 24.5-43.4%), followed by T. hydatigena (4.3%, 95% CI: 1.7-10.5%), *D. caninum* (2.2% 95%, CI: 0.6-7.5%%), and *T. polyacantha* (1.1%, 95% CI: 0.2-5.8%). Also, mixed infections were demonstrated in 13.6% of foxes (*Mesocestoides* + *T. polyacantha*) and 5.8% of jackals (*Mesocestoides* + *T. hydatigena*). Overall, cestode infection rate in wild carnivores was 48.4%. Molecular analysis with universal primers amplified a 450-bp fragment of the mitochondrial cox1 gene. Sequencing also demonstrated 100% and more than 99% identity between the sequences of *T. hydatigena* (n = 8) and *T. polyacantha* (n = 4) isolates and their corresponding GenBank reference sequences. This study represents the third report and the first molecularly confirmation of *T. polyacantha* in foxes in Iran. These findings emphasize the importance of monitoring cestode infections in wild canids for epidemiological and public health purposes.

Keywords

Cestode, Wild canids, Taenia polyacantha, Taenia hydatigena, Iran

Abbreviations

No Abbreviations

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Introduction

The Taenidae family comprises prevalent gut-dwelling helminths that develop from cystic stages, affecting various tissues of intermediate hosts and incurring financial costs for treatment in humans or organ/carcass condemnation in the livestock industry [1]. They are the most prevalent tapeworms in the small intestine of wild canines, such as dogs, jackals, wolves, and foxes [2]. Taenia species exhibit a predator-prey life cycle involving two mammals: one acts as the definitive host and the other as the intermediate host. Several Taenia species have previously been reported in Iran, including *Taenia hydatigena*, *Taenia pisiformis*, *Taenia polyacantha*, *Taenia multiceps*, *Taenia taeniaeformis*, *Taenia serialis*, and *Taenia ovis* [3, 4, 5, 6, 7, 8, 9, 10]. Among these, *T. hydatigena*, *T. pisiformis*, *T. polyacantha*, *T. multiceps*, *T. serialis*, and *T. ovis* are commonly found in the intestinal tract of canids [11, 12]. *T. hydatigena* is one of the most widespread taeniid cestodes infecting domestic and wild animals. Dogs, foxes, wolves, and cats act as definitive hosts. Metacestodes develop into large, pear-shaped cysticerci (*Cysticercus tenuicollis*) in the abdomen and organs of livestock, acting as intermediate hosts [13]. Another taeniid species, *T. polyacantha*, is found in canids across the Northern Hemisphere, especially in arctic foxes (e.g., *Alopex lagopus*) and red foxes, as well as dogs and wolves in southern regions. Rodents (e.g., *Microtus oeconomus*) serve as intermediate hosts, where the tapeworm resides in their peritoneal cavity or pleural space [14]. In *T. multiceps* infection, the larvae (*Coenurus cerebralis*) typically parasitize the central nervous system in livestock, causing neurological signs [15].

Apart from the genus *Taenia*, cestodes of the genera *Diplydium* and *Mesocestoides* are also common in wild canids. The flea tapeworm, *Diplydium caninum*, follows a heteroxenous cycle, involving definitive hosts (carnivores and sporadically humans) and intermediate hosts such as fleas and chewing lice of dogs and cats. Adult stages in wild and domestic carnivores may render symptoms, including diarrhea, scooting behavior, dullness, weight loss, anorexia, and poor hair coat [16]. Members of the genus *Mesocestoides*, possess a more complex life cycle involving multiple intermediate hosts, including rodents, amphibians, birds, and reptiles. Their larval stage (tetrathyridia), may lead to life-threatening peritonitis in animal hosts. Zoonotic infections in humans are usually caused by consuming raw/undercooked snake, chicken, or wild game viscera [17]. Although numerous Taenia species possess distinct morphological characteristics, several exhibit striking similarities, necessitating molecular analysis for effective differentiation [18, 19]. Various mitochondrial and nuclear genetic markers are used in the molecular analysis of cestodes, with a primary focus on diagnostic applications [20, 21]. Among the genes studied, mitochondrial genes (cox1 and nadh1) have proven valuable for genetic characterization of Taeniidae cestodes [18, 22].

Given the limited nationwide information on the molecular characterization of large *Taenia* spp. in wild carnivores in Iran, the present study was conducted to determine the morphometric and molecular analysis of large Taenia spp. in the small intestine of road-killed wild canids (jackal, fox, and wolf) along with the intestinal cestodes infection in the northern, northeastern, and northwestern regions of Iran.

Therefore, the aim of the present study was to investigate the occurrence, species diversity, and morphometric and molecular characteristics of intestinal cestodes in road-killed wild canids (jackals, foxes, and wolves) from northern, northeastern, and northwestern Iran. To the best of our knowledge, this study also reports the first molecular identification of Taenia polyacantha in wild canids in Iran.

Results

Epidemiological findings

Based on our results, 33 (47.8%) jackals and 12 (54.5%) foxes were infected with at least one tapeworm species. No infection was found in the two examined wolves. *Mesocestoides* spp. was the most prevalent cestode identified in both jackals and foxes. Among jackals, the overall prevalence of *Mesocestoides* spp. was 36.2%, including seven cases from northeastern Iran, seven from northwestern Iran, and fifteen from northern Iran. In foxes the overall prevalence of *Mesocestoides* spp. was 27.3%, including two cases from northeastern Iran, and seven from northwestern Iran. Other cestode species identified included: *T. hydatigena* (two foxes from northwestern Iran; and six jackals, two from northeastern Iran, and four from northwestern Iran), *T. polyacantha* (four foxes from northwestern Iran), and *Dipylidium* spp. (two jackals, one from northern Iran and one from northeastern Iran). Moreover, this study reports the first molecular identification of *T. polyacantha* in Iran. All four infected foxes were collected from Ardabil Province (Pars Abad, Qosabeh, and Meshgin Shahr cities) in northwestern Iran. Three of the four specimens (13.6%) were mixed infections with *Mesocestoides* spp. In addition, four jackal carcasses (5.8%) were found to be infected with *Mesocestoides* spp. and *T. hydatigena*. Among foxes, an equal number of males and females (n = 6) were infected with tapeworm species. In contrast, most infected jackals were female (n = 22; 41.5%). Most jackal and fox carcasses were collected from northernern (n = 35) and northwestern (n = 13) Iran, respectively. There was no statistically significant

association between the tapeworm infection and host species ($p = 0.09$), sex ($p = 0.32$), or geographical area ($p = 0.26$) (Tables 1 & 2). Microscopic observations of the parasites are illustrated in Figure 1.

Table 1.
Number and percentage of the wild canids infected with single or mixed cestode infections.

Type of animal	Type of Parasite Count (%)									P-value
	Mesocestoides spp	*Taenia hydatigena*	*Taenia polyacantha*	*Dipylidium caninum*	*Mesocestoides spp + Taenia hydatigena*	*Mesocestoides spp + Taenia polyacantha*	Negative	Positive	Total	
Fox	6 (27.3)	2 (9.1)	1 (4.5)	0 (0)	0 (0)	3 (13.6)	10 (45.5)	12 (54.5)	22 (100)	0.09
Wolf	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	2 (100)	
Jackal	25 (36.2)	2 (2.9)	0 (0)	2 (2.9)	4 (5.8)	0 (0)	36 (52.2)	33 (47.8)	69 (100)	
Total	31 (33.3)	4 (4.3)	1 (1.1)	2 (2.2)	4 (4.3)	3 (3.2)	48 (51.6)	45 (48.4)	93 (100)	

Table 2.
Gender- and region-based prevalence of wild canids infected with tapeworms.

Parameters	Infection count (%)						Positive	Total	P-value
	Fox		Wolf		jackal				
Gender	Yes	No	Yes	No	Yes	No			
Male	6 (15)	4 (10)	0	1 (2.5)	11 (27.5)	18 (45)	17 (42.5)	40 (100)	0.32
Female	6 (11.3)	6 (11.3)	0	1 (1.9)	22 (41.5)	18 (34)	28 (52.8)	53 (100)	
Total	12 (12.9)	10 (10.8)	0	2 (2.1)	33 (35.5)	36 (38.7)	45 (48.4)	93 (100)	
Region									
North	0 (0)	0 (0)	0	0 (0)	16 (45.7)	19 (54.3)	16 (45.7)	35 (100)	0.26
Northeast	2 (7.1)	7 (25)	0	2 (7.1)	9 (32.2)	8 (28.6)	11 (39.3)	28 (100)	
Northwest	10 (33.3)	3(10)	0	0 (0)	8 (26.7)	9 (30)	18 (60)	30 (100)	
Total	12 (12.9)	10 (10.8)	0	2 (2.2)	33 (35.4)	36 (38.7)	45 (48.4)	93 (100)	

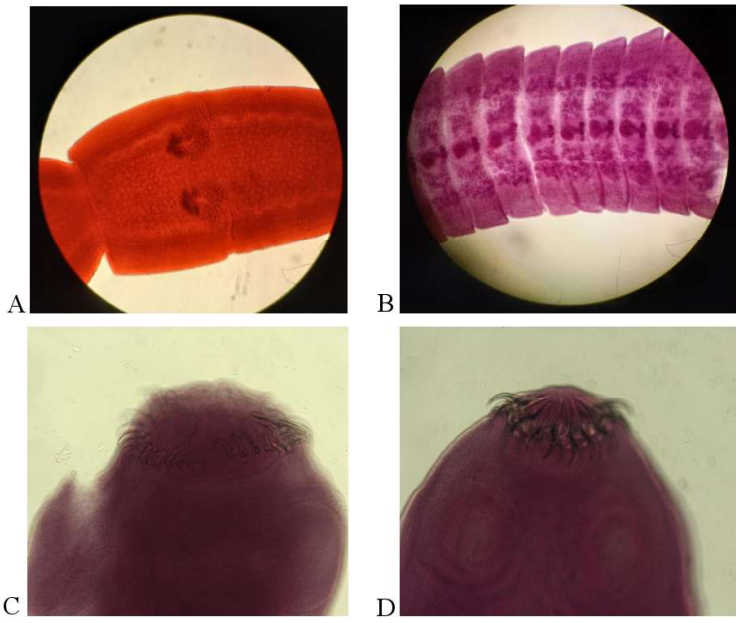

Figure 1.
Microscopic images of the parasites. (A) *Dipylidium caninum* (B) *Mesocestoides spp* (C) *Taenia polyacantha* (D) *Taenia hydatigena*.

Hook morphometry

The morphometry of small and large hooks was measured in *T. hydatigena* and *T. polyacantha*, which are primarily found in northwestern Iran. For *T. hydatigena*, the mean estimates of small hooks were 127.85 µm for TL, 55.62 µm for TW, and 58.5 µm for BL. The corresponding mean values for large hooks were 194.4 µm for TL, 59.28 µm for TW, and 90.52 µm for BL. In *T. polyacantha* the mean measurements of small hooks were 102.96 µm for TL, 43.19 µm for TW, and 65.03 µm for BL, whereas large hooks showed a mean of 182.33 µm for TL, 44.39 µm for TW, and 74.34 µm for BL. Among *T. hydatigena* isolated from a female fox, small hook estimates were higher than for the other isolates. Regarding large hooks, the highest TL (209.8 ± 4.88 µm) was recorded in *T. hydatigena* isolated from a male jackal, and the highest TW (66.77 ± 1.81 µm) was observed in *T. hydatigena* isolated from a female fox, both originating from the northwestern Iran. Also, the highest BL (111.3 ± 6.84 µm) was observed in a *T. hydatigena* specimen isolated from a male jackal in northeastern Iran. The highest TL (108.3 ± 1.35 µm) and TW (44.4 ± 3.14 µm) in the small hooks of *T. polyacantha* were recorded in a male fox, while the highest BL (66.38 ± 4.2 µm) was observed in another *T. polyacantha* specimen isolated from a different male fox. In addition, the highest TL (187 ± 1.22 µm) and TW (48.6 ± 4.34 µm) among the large hooks of *T. polyacantha* were recorded in a female fox, while the highest BL (75.15 ± 2.89 µm) was observed in a male fox. Other estimated hook lengths are tabulated in Table 3. Also, quantitative measurements of hooks dimensions and arrangement are provided in Figure 2.

Table 3.
Details of hook morphometry in isolated tapeworms from wild canids

Isolated Species	Host	Region	Small hooks						Large hooks					
			TL	ACL	PCL	BL	HL	TW	TL	ACL	PCL	BL	HL	TW
T. hydatigena	*Female fox*	*Northwest*	134.6 ± 8.57	70.85 ± 3.49	77.68 ± 9.23	63.85 ± 1.91	52.3 ± 9.63	65.85 ± 3.07	185.1 ± 2.99	93.0 ± 2.58	107.9 ± 2.3	81.57 ± 1.35	85.17 ± 2.49	66.77 ± 1.81
T. polyacantha	*Female fox*	*Northwest*	100.2 ± 1.24	65.2 ± 3.25	56.33 ± 3.85	62.53 ± 5.11	26.2 ± 3.2	42.37 ± 1.64	187 ± 1.22	90.27 ± 2.75	104.8 ± 1.5	75.1 ± 4.62	90.37 ± 6.32	48.6 ± 4.34
T. hydatigena	*Female jackal*	*Northwest*	121.1 ± 5.26	65.9 ± 2.73	67.33 ± 2.52	53.15 ± 2.62	51.4 ± 5.92	45.4 ± 3.64	188.5 ± 4.54	88.9 ± 5.76	107 ± 1.8	78.7 ± 3.01	89.93 ± 3.02	52.7 ± 6.29
T. hydatigena	*Male jackal*	*Northeast*	-	-	-	-	-	-	209.8 ± 4.88	117.5 ± 7.21	103.4 ± 2.36	111.3 ± 6.84	72.4 ± 5.85	58.37 ± 6.16
T. polyacantha	*Male fox*	*Northwest*	108.3 ± 1.35	74.4 ± 1.73	50.33 ± 2.3	66.2 ± 1.47	31.6 ± 4.09	44.4 ± 3.14	187 ± 0.21	91.05 ± 0.35	104.9 ± 0.35	75.15 ± 2.89	88.8 ± 5.09	44.65 ± 1.76
T. polyacantha	*Male fox*	*Northwest*	100.4 ± 0.88	72.58 ± 4.02	50.9 ± 2.84	66.38 ± 4.2	29.8 ± 2.82	42.8 ± 1.59	173 ± 8.31	85.87 ± 6.56	96.13 ± 5.83	72.77 ± 4.36	83.1 ± 5.4	39.93 ± 7.91

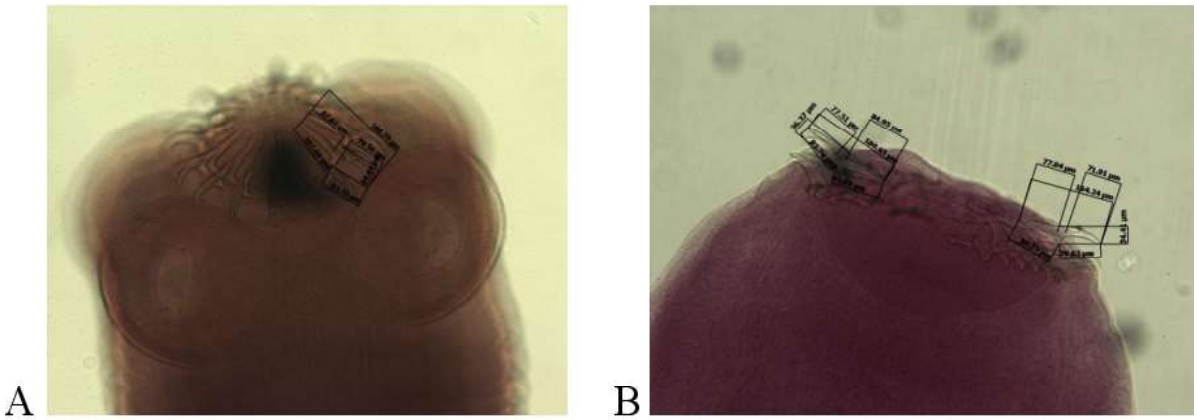

Figure 2.
Morphometric analysis of hooks. (A) *Taenia hydatigena* (B) *Taenia polyacantha*

A 450 bp fragment of mitochondrial COX1 was amplified (Figure 3), followed by DNA sequencing of Twelve isolates. Multiple sequence alignment analysis of *T. polyacantha* isolates with the standard isolate demonstrated a 100% nucleotide identity with the reference sequence available in GenBank (Accession No. EU544581.1). Similarly, more than 99% identity was obtained between *T. hydatigena* isolates in the present study with the registered GenBank sequence (Accession: MN478491.1); of note, single nucleotide substitutions were observed in position 261 (G → A) of *T. hydatigena* Ca13 and Ca14 isolates as well as in position 342 (T → C) of Ca6 isolate (Supplementary File). Phylogenetic analysis based on cox1 gene revealed that all four *T. polyacantha* isolates (isolates 6, 5, 13, and 14) were genotically identical to the Finnish genotype, as evidenced by multiple alignment. Also, they clustered within the other Finland isolates at the same branch. In contrast, *T. hydatigena* genotypes obtained in the present study (isolates 1, 2, 4, 7, 8, 9, 10, and 11) reported from wild canids clustered with reference sequences from Iraq (MZ313985), Slovakia (MZ345600, MZ298783), and Iran, Chabahar region (MN478491). More details on the phylogenetic relationship of the obtained isolates are illustrated in Figure 4. Deposition of DNA sequences for *T. polyacantha* isolates (Accession numbers: PP658436 (Vu1), PP658437 (Vu19), PP658443 (Vu4), PP658444 (Vu13)) and for *T. hydatigena* isolates (Accession numbers: PP658433 (Vu16), PP658434 (Ca2), PP658435 (Ca6), PP658438 (Ca10), PP658439 (Ca13), PP658440 (Ca14), PP658441 (Ca15w), PPP658442 (Ca15e)) was done in GenBank.

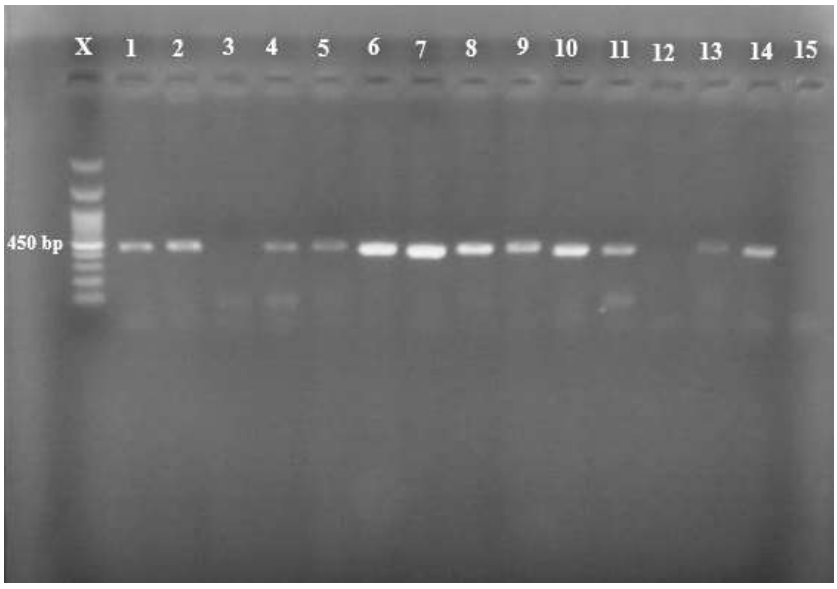

Figure 3.
X: DNA ladder 100bp, 1-15: Amplicon of mitochondrial cox1 gene (450 bp) using JB3 and JB4.5 primer.

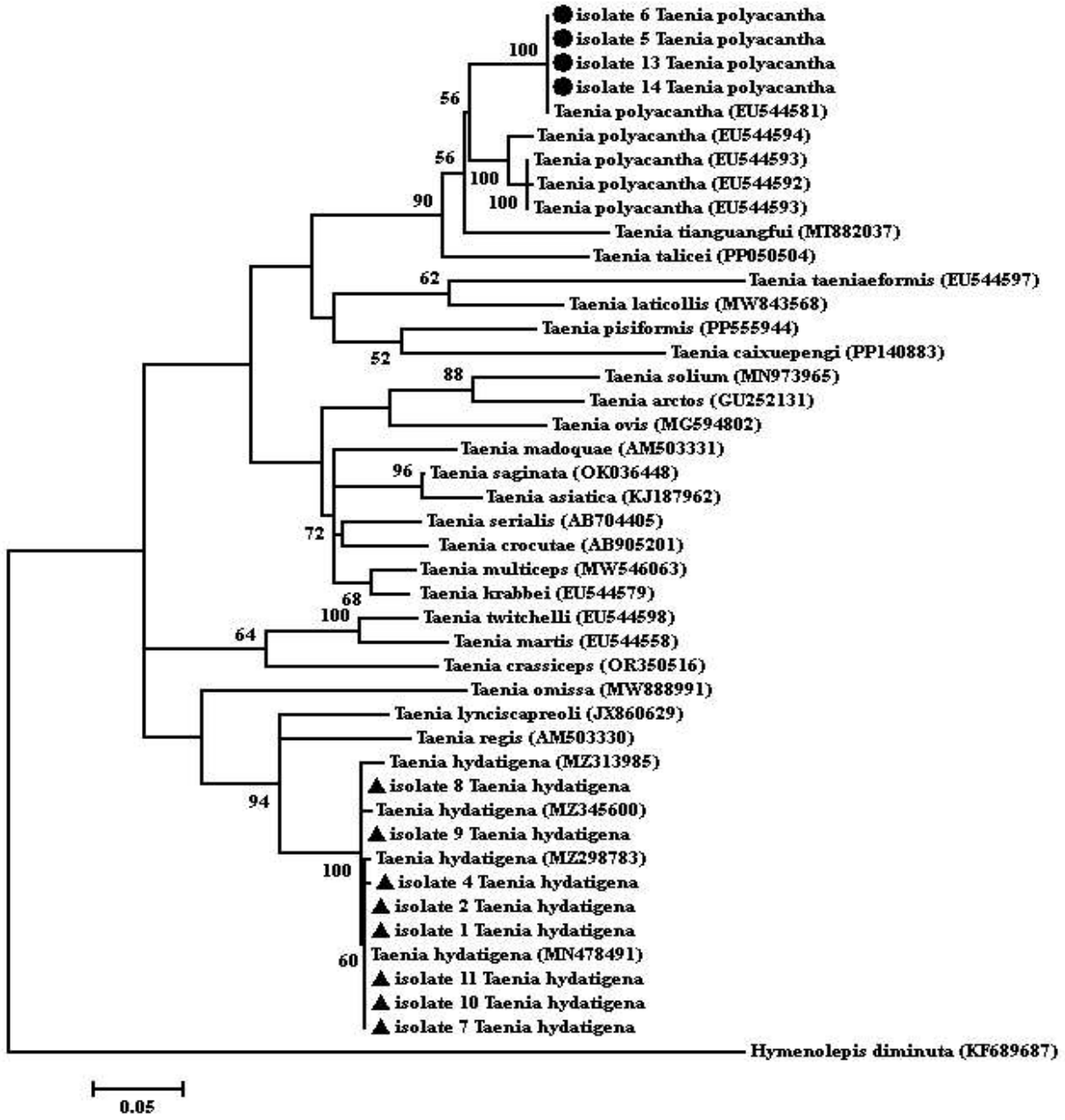

Figure 4.
Phylogenetic analysis of cestodes sequences obtained in the present study with those from other regions of the world. *Hymenolepis diminuta* was used as an outgroup. Iranian isolates of *T. polyacantha* (n = 4) clustered with Finland genotypes, while *T. hydatigena* isolates (n = 8) clustered with some genotypes from Iran, Iraq, and Slovakia in eastern Europe.

Discussion

Free-roaming jackals and red foxes can be a potential source of cestode infections, which are significant for both domestic animals and humans [23]. The present study assessed cestode infections in road-killed wild canids (n = 93, 69 jackals, 22 foxes, 2 wolves) from the northern territories of Iran. Our results determined four cestode species (*T. hydatigena*, *T. polyacantha*, *D. caninum*, and *Mesocestoides* sp.). The tolal prevalence of cestodes was 48.4%, with Mesosestoides spp representing the most common taxon (33.3%), followed by *T. hydatigena* (4.3%), *D. caninum* (2.2%), and *T. polyacantha* (1.1%). Although a higher prevalence rate was noted in foxes (54.5%) compared to jackals (47.8%), with *Mesocestoides* single infections predominating in jackals (36.2%) and foxes (27.3%). Intestinal helminths represent a significant portion of the worm burden in carnivore populations in Iran, accounting for 80.4% (95% confidence interval [CI]: 70.2% - 88.8%). Among these, jackals (97.32%) are the most heavily parasitized, followed by foxes (89.17%) and dogs (57.89%). Tapeworms are among the most common intestinal helminths found in domestic and wild canids in Iran, including *D. caninum* (20.45%),

T. hydatigena (15.28%), *M. lineatus* (11.83%), and *E. granulosus* (10%) [15]. Several studies have been conducted to document the cestode fauna in the canine population in Iran. In a nationwide investigation, red foxes and golden jackals from three different climatological zones in Iran were necropsied, representing a high predominance of *Mesocestoides lineatus* in jackals (15.2%-21.5%) and foxes (43.2% - 67.5%) [24]. In a focal study in the Moghan plain, northwestern Iran, of 85 necropsy specimens from wild canids, 96.47% were infected; among these, *Mesocestoides* (84.7%) was the most prevalent, followed by Joyuxiella (34.1%) and Taenia spp. (11.8%), and *D. caninum* (1.2%) [10].

In 63 intestinal specimens from dogs and jackals in Mazandaran province, northern Iran, a 95.2% prevalence of parasitic infection was observed, with 33.3% and 25.3% prevalence for *Mesocestoides* spp. and *D. caninum*, respectively [25]. In another study on 274 fecal samples from wild canids in this area, 85.2% were infected, and the ova of *D. caninum* was mostly found in 25.2% of dogs and 11.1% of jackals [26]. In a study on road-killed carnivores in the Caspian Sea littoral (2017), *D. caninum* (33.3% in dogs, 27.2% in jackals), *E. granulosus* (25.9% in dogs, 27.2% in jackals), and *T. hydatigena* (18.5% in dogs, 18.1% in jackals) were the most frequent isolated tapeworms; they also found less frequent cestodes, including *Mesocestoides, Joyuxiella,* and *Spirometra* (7.4%) [3]. In the present study, we also examined the small intestine of 2 wolves and it is noteworthy to say that they were without evidence of cestode infections. In Mazandaran, 5.2% of 19 wolves were infected with Taenia species [26]. Additionally, numerous studies worldwide have reported various cestode species in wolves [27, 28, 29].

In a similar study in Turkey (2021), *Mesocestoides* sp. and *D. caninum* were the most and least prevalent cestode species isolated from foxes [30]. Consistent with our findings, the pooled prevalence of *Mesocestoides* infection in terrestrial carnivores was estimated to be 21.72% (95% CI: 18.49% - 25.14%) [17]. In comparison, the North American dogs had the lowest rates (0.054%) [31]. Infection with adult worms was more pronounced in foxes [35.97% (95% CI: 29.54% - 42.66%)] and other wild canids [21.39% (95% CI: 15.25% - 28.24%)] than in domestic carnivores such as dogs and cats, highlighting the role of feeding behavior in the maintenance of parasite population; wild canids have free access to potentially infected intermediate hosts (e.g., rodents, birds, snakes, amphibians) harboring tetrathyridia. The authors also remarked a 28% (95% CI: 19.83% - 36.97%) prevalence of *Mesocestoides* infection in Iran [17]. Our finding regarding the dominance of *Mesocestoides* in the canine population in Iran overlaps with previous studies, whereas it contrasts with the Eslahi et al. study, which demonstrated *D. caninum* as the most prevalent tapeworm in road-killed carnivores in northern Iran [3]. We also demonstrated mixed infections of *Mesocestoides* sp. with *T. hydatigena* (5.8%) in jackals and with *T. polyacantha* (13.6%) in foxes.

The significant findings of the present study was the isolation and of *T. polyacantha* from fox populations, and the molecular confirmation of this species in Iran for the first time. Although, this is the third report of *T. polyacantha* in Iran, while in the previous studies in Iran molecular identification was not performed; the first documentation dates back to 1973, when Mobedi et al. isolated a *T. polyacantha*-like worm from 22.5% and 2.5% of red foxes in Kalibar and Moghan regions, northwestern Iran [5]. The second report was by Zare-Bidaki (2010) again in red foxes in the Moghan plain (6 recovered worms) [10]. Based on the reports above, it can be concluded that the Moghan Plain, along with the semi-arid mountainous areas of northwestern Iran with well-developed vegetation, represents favorable ecological niches for the development and propagation of *T. polyacantha* cestodes in red foxes, and potentially in their respective intermediate hosts. Various fox species (e.g., Vulpes and Alopex) are recognized as definitive hosts for adult *T. polyacantha*, acquiring infection through predation on infected small mammals, such as lagomorphs and voles [32] as a substantial part of red fox diets [33] The metacestodes cause fibrosarcoma, hepatitis, and peritonitis in affected hosts. Generally, canids in northern Eurasia and southern tundra ecosystems may harbor this taeniid species. Nevertheless, dogs and wolves in more southern areas may also harbor this tapeworm [34, 35, 36]. In the present study, *T. polyacantha* was found in 1/22 examined foxes (2.2%); mixed infections with *Mesocestoides* sp. were observed in 3/22 (13.6%) of foxes. This finding is consistent with that of Bouchard and colleagues (2021), who documented *T. polyacantha* in 3/176 (2%) of foxes in Canada [37]. Also, a similar trend was observed in 197 red foxes in mid-Wales, with a 4% prevalence of *T. polyacantha*. In contrast, this cestode species was found in 166/269 red foxes (61.7%) during post-mortem examination in Lithuania, eastern Europe [38]. In the neighboring country, Turkey, *T. polyacantha* has been reported to occur at a prevalence of 13.6%-15.53% [30]. However, a highly variable prevalence rate of 1.1% to 70.4% has been reported for *T. polyacantha* globally [39, 40, 41]. The adult worm is equipped with 44-68 rostellar hooks; the TL of the large hooks discovered in the present study was within the range of previous iso-

lates (178-234 µm), whereas our isolates demonstrated shorter small hooks than previous ones (120-182 µm). Hook length is not reliable in differentiating *T. polyacantha* from other sympatric species (*T. hydatigena* and *T. crassiceps*); however, the former has more hooks with somewhat distinctive shapes [14].

Another finding of the present study was the isolation and molecular characterization of *T. hydatigena* in both foxes (n = 2; 9.1%) [34] and jackals (n = 2; 2.9%). The low prevalence observed in the present study is consistent with the study done by Meshgi et al. (2010) in various regions in Iran [24] and with the study done by Bouchard et al. (2021) in wild canids in Canada [37]. Nevertheless, this taeniid species has previously shown moderately high prevalence rates in Iran, such as northern areas (18.5% Guilan [3], ~30% Mazandaran [42]) and eastern parts (45% Sistan region [43], 43% Mashahd [44]) as well as in other countries such as Uzbekistan (57.4% in dogs) [45] and Kazakhstan (21.7% in red foxes) [46]. Definitive hosts acquire this cosmopolitan infection through ingestion of infected domestic livestock, particularly sheep carcasses harboring *C. tenuicolis* metacestodes. This metacestode can render mortality in young animals and cause significant organ damage, mainly by affecting the liver, omentum, mesentery, peritoneum, pleura, and other internal viscera [47, 48].

Since taeniid species possess homologous adult and egg morphological characteristics, they should be differentiated using molecular techniques. In this sense, different PCR-based and sequencing approaches have been developed. Mitochondrial DNA is particularly valuable because of their rapid evolutionary rate and high copy number, and it is of particular interest for the molecular identification of closely related organisms [49]. The mitochondrial cox1 gene is one of the well-known genetic markers for cestode differentiation [50]. In the present study, a 450 bp cox1 fragment of this marker was amplified using universal primers (JB3, JB4.5), followed by sequencing of four *T. polyacantha* and eight *T. hydatigena*. Of note, three specimens isolated from northwestern Iran were negative in repeated PCR analyses; this could be due to degraded DNA molecules during extraction. Our results indicated 100% and more than 99% identity in multiple alignments of the obtained partial cox1 gene sequences with the standard sequences previously deposited in GenBank.

This study confirms that *T. polyacantha* uses the red fox as its definitive host in Iran.

Phylogenetic analysis demonstrated that the Iranian isolates of *T. polyacantha* (n = 4) clustered with Finnish genotypes, while *T. hydatigena* isolates (n = 8) clustered with some genotypes from Iran, Iraq, and Slovakia in eastern Europe

Phylogenetic analysis indicated that *T. polyacantha* is closely related to *T. talicei* and more distantly related to *T. hydatigena*. These conclusions are corroborated by Gomez-Puerta [18]. Furthermore, *T. hydatigena* demonstrates a closer affinity to *T. lyncis-capreoli*, consistent with previous studies [51, 52].

Conclusion

The present study provides new insight into the molecular identification and phylogeny of Taenia species infecting wild canids in Iran. Most notably, it represents the first molecularly confirmation of *T. polyacantha* in Iranian foxes, indicating potential hotspots for the sylvatic circulation of this Taenia species.

Materials and Methods

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that no generative AI or AI-assisted technologies were used in the writing of this manuscript.

Ethical Considerations

During the study, proper precautions were taken to safeguard the researchers' health and safety. All procedures were conducted in accordance with the code of ethics (Ethical code: IR.UM.REC.1400.270).

Sample collection

This cross-sectional study involved necropsies of 93 road-killed wild canids collected between 2022-2024 in the country's northern, northeastern, and northwestern regions of Iran, including 69 jackals, 22 foxes, and 2 wolves. General characteristics such as location, date, animal species, sex, and age were documented for each animal.

Morphology and morphometry evaluation

Staining with acid carmine was performed after isolating the tapeworms from the wild canids' intestines [53, 54]. Species identification of the isolated cestodes was performed using established taxonomic key based on morphological and morphometric parameters, including the number and size of the hooks and the number of lateral uterus branches in the fertile proglottid. The following hook measurements were recorded in micrometers (µm): including total length (TL), anterior chord length (ACL), posterior chord length (PCL), blade length (BL), handle length (HL), and total width (TW). Morphometric analyses were conducted using AxioVision LE software. Measurements are presented as mean ± standard deviation (SD) [55, 56].

Molecular and phylogenetic analysis

DNA extraction was performed using a commercial DNA

extraction kit (MBST, Iran) to characterize Taenia species in accordance with the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop spectrophotometer, and extracted DNA samples were stored at -20 °C until analysis. The universal primers (JB3, JB4.5) amplifying the 450 base pair (bp) fragment of the Mitochondrial Cytochrome C Oxidase Subunit 1 gene (cox1) of the helminths were used, as follows: JB3: 5'-TTTTTTGGGCATCCTGAGGTTTAT-3' and JB4.5: 5'-TAAAGAAAGAACATAATGAAAATG-3' [57, 58]. Polymerase chain reaction (PCR) mixture was prepared in a 25 µl volume, containing 12.5 µl of MasterMix, 2 µl of each primer (10 pmol), 1 µl of extracted DNA sample, and 9.5 µl of distilled water (DW). The PCR reaction was done in a Bio-Rad thermal cycler device, as follows: an initial denaturation step at 94 oC for 5 min; 40 cycles of denaturation at 94 oC for 30s; annealing at 52 oC for 45s); and extension at 72 oC for 35s); followed by a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on 1.5% agarose, and 450 bp bands were visualized under ultraviolet (UV) illuminator. The obtained amplicons were sequenced using the Applied Biosystems™ 3500 (Life Technologies) The ClustalW program was used to edit and align the sequences, while BLAST algorithms and the National Center for Biotechnology Information databases were utilized for sequence analysis. The MEGA v6.03 software generated a maximum-likelihood phylogeny, with Hymenolepis diminuta as the outgroup (Accession No.: KF689687).

Statistical analysis

Statistical analyses were performed using IBM® SPSS® software (Version 21, Armonk, NY USA). The Chi-square test was used to assess parasite distribution by gender, geographic area, and examined canine species. Statistical significance was set at $p < 0.05$.

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Authors' Contributions

Elahe Ebrahimzadeh conceived and planned the experiments. Zahra Sharifi Darvazeh carried out the experiments. Moein Abolhasani Darounkola contributed to sample preparation. Hassan Borji contributed to the interpretation of the results. Zahra Sharifi Darvazeh took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Comparison of the Immunogenicity of Different Proportions of Chitosan Adjuvant in an Attenuated *Neospora caninum* Vaccine in BALB/c Mice

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ABSTRACT

Neosporosis is a neuromuscular infectious disease in dogs caused by the protozoan *N. caninum*. This disease is globally distributed; canines are the definitive hosts, while a wide range of animals serve as intermediate hosts. This study aimed to compare the immunogenicity of different proportions of chitosan adjuvant in an attenuated *N. caninum* vaccine in BALB/c mice. Twenty-eight BALB/c mice were divided into four groups (n = 7 per group). Groups 1, 2, and 3 were subcutaneously immunized with attenuated tachyzoites of *N. caninum* strain Nc-1 combined with micronized chitosan adjuvant at concentrations of 10%, 50%, and 90%, respectively. Group 4 served as the negative control and did not receive any vaccine formulation or adjuvant. All groups were monitored daily over a three-week period. Following sample collection, antibody levels were quantified using ELISA, and cellular immune responses were evaluated by measuring IFN-γ levels with a commercial assay kit. According to ELISA results, the highest antibody levels were observed in the group immunized with the attenuated *N. caninum* strain containing 90% chitosan adjuvant, showing significantly higher antibody levels compared to the control and other groups ($p < 0.05$). Similarly, IFN-γ analysis revealed that the strongest IFN-γ-associated immune response was observed in the group immunized with 90% chitosan adjuvant, with significant differences compared to the control and other groups ($p < 0.05$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Based on these findings and the considerable effect of chitosan adjuvant on both antibody levels and IFN-γ-associated cellular immunity, these findings support further investigation of chitosan-containing formulations as potential adjuvants for Neospora vaccine development.

Keywords

Neospora caninum, Chitosan, Vaccine adjuvant, Immunogenicity, BALB/c mice, IFN-γ

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Abbreviations

ANOVA: Analysis of Variance
(BALB/c): Bagg Albino laboratory mouse strain
DMEM: Dulbecco's Modified Eagle Medium
ELISA: Enzyme-Linked Immunosorbent Assay

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Introduction

N. caninum is a protozoan parasite transmitted transplacentally, with a wide range of intermediate hosts and canines as definitive hosts. In many countries, it is a leading cause of abortion in cattle and a significant cause of neuromuscular paralysis in dogs [1]. Cattle can be infected congenitally through placental transmission or postnatally by ingesting oocysts shed by the definitive hosts. Currently, there is no effective drug to treat infected cattle; therefore, prevention relies mainly on management and hygiene practices [2]. Developing an effective vaccine would greatly aid in controlling this disease.

Chitosan is a natural polysaccharide derived from the partial deacetylation of chitin. It possesses polymeric properties that vary according to its degree of deacetylation, molecular weight, viscosity, and the number of amino groups. These features enable chitosan to chemically interact with anionic systems, resulting in changes to its physicochemical properties. Consequently, chitosan exhibits excellent biological properties, including biodegradability in the human body, as well as immunological, antibacterial, and wound healing activities [3].

Chitosan has been extensively studied as a vaccine adjuvant due to its biocompatibility, biodegradability, and immunostimulatory properties. Previous studies have demonstrated that chitosan-based formulations can enhance both humoral and cellular immune responses against various pathogens. For instance, Guo et al. (2018) demonstrated that a chitosan microsphere vaccine significantly increased IFN-γ, IL-4, and IL-10 levels in mice immunized against Toxoplasma gondii [8]. However, limited research has been conducted on the use of chitosan as an adjuvant in Neospora caninum vaccines. While attenuated *N. caninum* vaccines have shown promise in inducing protective immunity [ref], the optimal adjuvant formulation for enhancing their immunogenicity remains to be established.

To our knowledge, no study has systematically compared the immunogenicity of different proportions of micronized chitosan when combined with an attenuated *N. caninum* vaccine. The concentrations of 10%, 50%, and 90% were selected based on a preliminary dose-finding study (data not shown) that evaluated a range of chitosan concentrations (5–95%) for compatibility with the vaccine formulation and absence of local toxicity. These three concentrations were chosen to represent a low (10%), medium (50%), and high (90%) proportion of chitosan in the final vaccine formulation, allowing for a systematic evaluation of the dose-response relationship.

We hypothesized that increasing the concentration of micronized chitosan adjuvant would enhance both humoral and cellular immune responses to the attenuated *N. caninum* vaccine in a dose dependent manner. Therefore, this study aimed to compare the immunogenicity of different proportions of chitosan adjuvant in an attenuated *N. caninum* vaccine in BALB/c mice.

Abbreviations-Cont'd

FBS: Fetal Bovine Serum
IFN-γ: Interferon-gamma
J774: Murine macrophage-like cell line OD: Optical Density
OPD: o-Phenylenediamine
PBS: Phosphate-Buffered Saline
PPLO: Pleuropneumonia-like Organism
RPMI: Roswell Park Memorial Institute medium Vero: African Green Monkey kidney cell line

Results

Evaluation of Vero cell line culture and passage

Visual inspection of the culture flasks indicated clear and transparent medium, suggesting no microbial contamination. Microscopic examination showed that Vero cells preserved their characteristic regular polyhedral morphology. Followin g inoculation, tachyzoite release accompanied by progressive cell destruction was observed within 4 to 7 days post-inoculation. The harvested tachyzoites obtained at peak release (yield range: 5.2×10^7 to 8.1×10^7 per flask, viability >95% by trypan blue exclusion) were subsequently used for immunization.

Assessment of microbial contamination in vaccine preparations

Cell culture monitoring over 48 hours showed no evidence of microbial contamination in the prepared vaccine formulations. Extended observation for 21 days confirmed the absence of Mycoplasma contamination in the cell lines used for vaccine production.

Immunization outcomes in experimental mice

To evaluate the immunogenic potential of the experimental formulations, both humoral and cellular immune responses were analyzed using ELISA and IFN-γ assays.

The ELISA results demonstrated that the group immunized with the attenuated *N. caninum* strain combined with 90% chitosan adjuvant (G3) exhibited the highest antibody levels. In contrast, Group G4 (control) displayed the lowest responses. Comparisons indicated that G3 induced significantly higher antibody levels compared to all other groups (vs. G1, $p = 0.0002$; vs. G2, $p = 0.0002$; vs. G4, $p < 0.0001$). No

significant difference was observed between Groups G1 and G2 (p = 0.9496). However, both G1 and G2 showed higher antibody levels than G4 (G1 vs. G4, p = 0.0474; G2 vs. G4, p = 0.0320). Analysis of IFN-γ levels further confirmed these trends. Group G3 induced the strongest IFN-γ-associated immune responses, which were significantly higher than those of G1 (p = 0.0003), G2 (p = 0.0002), and G4 (p < 0.0001). No significant difference was detected between G1 and G2 (p = 0.3333). In contrast, Group G4 exhibited a markedly weaker response compared to both G1 (p = 0.0005) and G2 (p = 0.0008).

Overall, these findings demonstrate that the 90% chitosan adjuvant formulation (G3) significantly enhanced both humoral and IFN-γ-associated immune responses compared to all other groups, highlighting its superior immunostimulatory potential. Group G4 (control) produced the weakest responses, while Groups G1 and G2 displayed intermediate and comparable levels of immunity (Table 1).

Table 1.
Pairwise comparisons of ELISA (OD450) and IFN-γ (pg/mL) responses between experimental groups.

Comparisons Groups	Mean Diff.		95.00% CI of diff.		Adjusted P Value	
	ELISA	Gamma interferon	ELISA	Gamma interferon	ELISA	Gamma interferon
G1 vs. G2	0.03550	-0.04500	-0.2419 to 0.3129	-0.1383 to 0.04828	0.9496	0.3333
G1 vs. G3	-0.2820	-0.3300	-0.5594 to -0.004647	-0.4233 to -0.2367	0.0474	0.0005
G1 vs. G4	1.262	0.3650	0.9846 to 1.539	0.2717 to 0.4583	0.0002	0.0003
G2 vs. G3	-0.3175	-0.2850	-0.5949 to -0.04015	-0.3783 to -0.1917	0.0320	0.0008
G2 vs. G4	1.227	0.4100	0.9491 to 1.504	0.3167 to 0.5033	0.0002	0.0002
G3 vs. G4	1.544	0.6950	1.267 to 1.821	0.6017 to 0.7883	0.0001	<0.0001

Data are presented as mean ± SD (n=7 per group). Group labels: G1 = 10% chitosan; G2 = 50% chitosan; G3 = 90% chitosan; G4 = negative control. P values adjusted for multiple comparisons using Tukey's post-hoc test.

Discussion

A review of the literature revealed that limited studies have investigated the effects of various adjuvants on antibody levels and cellular immunity titers, particularly in the context of *N. caninum* vaccines. Consistent with the findings of this study, the formulation containing chitosan was associated with significantly higher antibody levels in the tested samples. In addition, the chitosan-containing formulation exhibited a notable and statistically significant enhancement of IFN-γ-associated immune responses. Adjuvants are critical components of vaccines, as they not only enhance immunogenicity but are also safe for use in both humans and veterinary applications [7].

In the present study, the highest antibody levels were observed in the group immunized with the attenuated *N. caninum* strain combined with 90% chitosan adjuvant (G3). The results highlight the significant impact of the chitosan-containing formulation on IFN-γ-associated immune responses. Previous research has demonstrated that adjuvants can induce long-term humoral immunity, which is a key advantage in vaccine development. However, it is important to note that humoral immunity alone is often insufficient to protect against parasitic infections, which typically require robust cellular immune responses.

For instance, Guo et al. (2018) investigated the immunomodulatory effect of chitosan adjuvant in combination with a Toxoplasma gondii protein vaccine in mice. Their findings showed significant increases in IFN-γ, interleukin-4, and interleukin-10 levels, leading to the conclusion that chitosan may serve as an effective carrier system for toxoplasmosis vaccines [8]. While this is consistent with our findings in *N. caninum*, important differences between the two parasites (*T. gondii* vs. *N. caninum*), antigens, and vaccine formulations must be considered. Additionally, the present study used micronized chitosan rather than chitosan nanoparticles used in previous studies.

Cellular immunity plays a crucial role in protec-

tive immunity against neosporosis, primarily through the production and secretion of cytokines such as IFN-γ. This cytokine, produced by T cells, is essential for controlling *N. caninum* infection through the activation of intracellular parasite killing mechanisms. Studies have also shown that mice, as experimental models, have limited endogenous IFN-γ production, which can be significantly enhanced through the administration of adjuvants such as chitosan [9]. Consistent with these reports, the present study demonstrated that the chitosan-containing formulation significantly increased IFN-γ levels, thereby indicating enhanced Th1-associated cellular immune responses. Additionally, chitosan has been previously shown to promote Th2 immune responses in other studies (Singla & Chawla, 2001), which is consistent with the elevated antibody levels observed in the present study. However, the current study did not directly measure Th2-associated cytokines (e.g., IL-4, IL-10), and further studies are needed to confirm Th2 polarization.

These findings collectively underscore the importance of chitosan as a potent adjuvant capable of stimulating both humoral and IFN-γ-associated immune responses. Overall, the results of this study demonstrate a positive and significant effect of chitosan-containing formulations on the immune response against *N. caninum*. ELISA tests revealed that the group immunized with attenuated *N. caninum* vaccine combined with 90% chitosan adjuvant (G3) exhibited the highest antibody levels, significantly greater than those of the control and other groups ($p < 0.05$). Similarly, IFN-γ assays confirmed that this group mounted the strongest IFN-γ-associated immune response ($p < 0.05$).

While our findings demonstrate that the 90% chitosan formulation significantly enhances immunogenicity (antibody and IFN-γ responses), these results do not establish protective efficacy. Challenge studies with *N. caninum* infection are needed to determine whether the observed immune responses translate to clinical protection. Therefore, we recommend further investigation of chitosan-containing formulations in protective efficacy studies.

Limitations

Several limitations of this study should be acknowledged:

1- The sample size (n=7 per group) was relatively small, and findings should be validated in larger studies.

2- No vaccine-only (without chitosan) or chitosan-only groups were included, limiting our ability to isolate the independent adjuvant effect of chitosan versus the vaccine antigen.

3- No challenge infection was performed, so the protective efficacy of the observed immune responses could not be evaluated.

4- Assessment was limited to ELISA and IFN-γ; direct cellular immune phenotyping (CD4/CD8, lymphocyte proliferation) was not performed.

5- The attenuated vaccine was validated by reduced virulence, but detailed characterization of attenuation stability was limited.

6- The study duration (three weeks post-immunization) does not address long-term immunogenicitted to 2×10^6 tachyzoites per mouse.

Materials and Methods

Declaration of Generative AI and AI-assisted technologies in the writing process

Artificial intelligence has been used to improve the grammar of the English text.

Ethical approval

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Karaj Islamic Azad University under the ethical approval Code IIR. IAU.K.REC.1402.43. for continued incubation.

Mycoplasma contamination testing

Due to the risk of Mycoplasma contamination in cell cultures, all cultures were tested using PPLO broth and agar media. Samples were incubated in PPLO broth (pH 7.6–8) at 37°C for 24–48 hours. Subsequently, 0.2 mL of each sample was inoculated onto PPLO agar (3.5%, pH 6.7–8) and incubated at 37°C for 21 days. Cultures were monitored for colony growth and pH changes. Escherichia coli (ATCC 25922) was used as a positive control on blood agar to verify that the culture medium supported bacterial growth and that the detection system was functional. Sterility of the vaccine preparations was confirmed by absence of bacterial growth on blood agar and absence of Mycoplasma growth on PPLO agar after 21 days.

Study animals and vaccination

A total of twenty-eight female BALB/c mice (6–8 weeks old, weight 18–22 g) were obtained from [source]. Animals were housed under standard conditions (22 ± 2°C, 12:12 light/dark cycle, ad libitum food and water) with 4 mice per cage. Mice were randomly divided into four groups (n=7 per group) using a computer-generated random number sequence (Microsoft Excel, RAND function). Group allocation was performed by an investigator not involved in the animal care or outcome assessment. Groups 1, 2, and 3 were subcutaneously injected with 2×10^6 attenuated *N. caninum* tachyzoites (strain Nc-1) combined with micronized chitosan adjuvant (molecular weight: [X] kDa, degree of deacetylation: [Y]%, particle size: [Z] µm, supplier: [Company]) at concentrations of 10%, 50%, and 90% (w/v of final formulation), respectively. Group 4 served as the negative control and did not receive any vaccine formulation or adjuvant. Each mouse

received a single 100 µL subcutaneous injection in the dorsal neck region. Animals were monitored daily for three weeks to assess their general health status (body weight, behavior, injection site reaction, and clinical signs). At the end of the experimental period (3 weeks post-immunization), blood samples were collected from all mice via cardiac puncture. Serum was separated and stored at -20°C until subsequent immunological analyses. The laboratory technician performing the ELISA and IFN-γ assays was blinded to the group assignments until all data were collected.

Antigen preparation and ELISA test

Neospora antigen was prepared by sonicating 2×10^9 tachyzoites in 1 mL of phenylmethylsulphonyl fluoride (2 mM) on ice. The mixture was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was used as the antigen. For ELISA, microtiter plates were coated with 100 µL of antigen (10 µg/well) diluted in bicarbonate buffer, and incubated at 4°C for 24 hours. Plates were then blocked with 3% bovine serum albumin in PBS-Tween (0.05%) for 2 hours at room temperature.

After washing, mouse serum samples diluted 1:100 were added and incubated at 37°C for 1 hour. Plates were washed, and 100 µL of anti-mouse HRP-conjugated IgG (diluted 1:2000) was added and incubated at 37°C for 1 hour. After washing, 100 µL of OPD substrate was added and incubated in the dark for 15 minutes, then the reaction was stopped with 100 µL of 12.5% sulfuric acid. Absorbance was read at 450 nm using a microplate reader [6]. All samples were run in duplicate, and the mean absorbance was used. Blank wells (substrate only), negative serum control (from unimmunized mice), and positive serum control (from mice previously immunized with *N. caninum*) were included on each plate.

Gamma interferon (IFN-γ) assay

Serum IFN-γ levels were quantified using a commercial mouse IFN-γ ELISA kit (Invitrogen™ Mouse IFN-γ ELISA Kit, catalog # [X], Thermo Fisher Scientific, Waltham, MA, USA) based on monoclonal antibodies, following the manufacturer's protocol. The detection range was 15.6–1000 pg/mL with a sensitivity of <5 pg/mL. The required sample volume was determined according to the

Resource Equation Approach with E value = 24 (total animals 28, groups 4, so error df = 24). Samples were run in duplicate.

Data analysis

Statistical analysis was performedusing SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare group means, followed by Tukey's post-hoc test for multiple pairwise comparisons. Adjusted P values were calculated using Tukey's method. A p-value of less than 0.05 was considered statistically significant. Data are presented as mean ± SD unless otherwise stated. Each mouse was considered as one experimental unit.

Authors' Contributions

Seyed Reza Hosseini and Marzieh Kefayat planned the experiments. Milad Hamzehali Tehrani and Amirhosein Norouzi took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Competing Interests

The authors declare that there is no conflict of interest.

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Study of major causes and associated economic losses of carcass and organ condemnation in cattle and sheep slaughtered at two abattoirs in the north of Algeria

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ABSTRACT

Inspection at slaughterhouses is an important step for providing a safe meat for human consumption. A cross-sectional study, involving antemortem and postmortem inspection was conducted from January to April 2025 to establish condemnation reason in cattle and sheep slaughtered and inspected at two slaughterhouses of Blida, northern Algeria. A total of 1091 animals, comprising 805 sheep and 286 cattle, slaughtered during the period of the present study, 14.78% sheep and 32.51% cattle were condemned. Among the animals examined, the most dominant condemnation patterns were pneumonia (38.65%), hydatidosis (16.80%), abscess (15.96%) and respiratory strongylosis (15.96%) for sheep and tuberculosis-compatible lesions (52.68%), hydatidosis (15.05%), abscess (11.82%), and pneumonia (10.75%) for cattle. Condemnation rate of lungs, hearts, livers, and heads for sheep were 84.87%, 2.52%, 7.56% and 4.20% respectively and for cattle were 91.39%, 49.46%, 26.88%, 1.07% respectively. Also, 4.30% carcasses, 3.22% stomachs and 1.07% udders were condemned only in cattle. Main causes of lung condemnation were tuberculosis-compatible lesions (56.47%), hydatidosis (14.11%), pneumonia (11.76%), abscesses (7.05%), and emphysema (7.05%) for cattle, whereas pneumonia (45.54%), abscesses (18.81%), hydatidosis (13.86%), hemorrhagic lung (7.92%), and emphysema (5.94%) for sheep. Liver was condemned mainly associated with abscess (36%), hydatidosis (32%) and tuberculosis-compatible lesions (24%) in cattle, while hydatidosis (55.55%), hepatitis (33.33%) and abscesses (11.11%) were the main causes in sheep. For the hearts, tuberculosis-compatible lesions (97.82% for cattle and 66.66% for sheep) was the major cause of condemnations. The total estimated economic losses was **30610.00** USD. Improving slaughterhouse facilities and conditions would facilitate the work of veterinary inspectors. Similarly, analyzing the collected data would enable the development of action plans to reduce the prevalence of specific pathologies.

Keywords

Abattoir, cattle, food safety, condemnation, sheep

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Abbreviations

FAO : Food and Agriculture Organization
Cis : Confidence intervals
LSD:Lumpy Skin Disease
TRP : Traumatic reticuloperitonitis

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Introduction

Meat has high nutritional value, particularly because its rich in high-quality proteins, minerals, and other essential nutrients [1]. Ensuring the safety and wholesomeness of meat is very important for protecting public health [2]. Abattoirs play an important role, in surveillance of various diseases of public and veterinary health importance [3]. The collection of sanitary information at slaughterhouse level contributes to improving knowledge of the national epidemiological situation [4]. During ante mortem and post mortem inspection, organs and carcasses may be condemned due to various pathogens and /or pathological conditions [5]. The main causes of organ condemnation during post mortem inspection are diseases originated by parasites, bacteria and viruses [6]. Some of the pathogens are zoonotic and therefore have public health significance ; while and all causes of condemnation incur serious economic losses to the livestock industry [5].

Many studies have been carried out at the slaughterhouse level to determine the prevalence and to collect information on reasons for meat and offal condemnation [7, 8]. Reported condemnation rates vary from 0.02% [9] to 87.7% [1] and the data about the main causes of carcass and organ condemnation in different countries varies significantly [10, 11].

In the Blida region of northern Algeria, livestock farming is focused on cattle production and sheep fattening. Also, the consumption of red meat has increased with the growth of the local population. The number of cattle slaughtered in this region increased from 6,762 animals in 2021 to 27,752 in 2024, while the number of sheep slaughtered has been estimated at 106,260 in 2024 [12]. Currently, there is a limited information on occurrence of various diseases/causes and their economic losses due to organ and carcass condemnation in cattle and sheep in the north of Algeria specially the Blida region. Although similar studies have been conducted in other regions [13, 14], it is important to carry out the study because the prevalence and diseases occurrence may differ according to geographical areas. The aim of the present study is to determine the causes of organ and carcass condemnations in cattle and sheep based on ante-mortem and post-mortem inspection procedure at two abattoirs in the Blida region of northern Algeria and to evaluate the associated economic losses.

Results

Number and percentage of condemnations in slaughtered animals

A total of 212 animals were condemned for various reasons, correpresenting to a prevalence of animals with at least one condemned organ or carcass of 19.43% (95%CI: 17.1% - 21.8%). The prevalence of condemnations were higher in cattle than in sheep ($p < 0.00001$) (Table 1).

Table 1.
Number and percentage of condemnations in slaughtered animals

Animal species	Number examined	Condemnations n (%)	*95% CI*
Sheep	805	119 (14.78)32.51)	[12.3% - 17.2%]
Cattle	286	93 (32.51)	[27.1% - 37.9%]

95% CI (%) :95% confidence interval

Number and percentage of condemnations according to sex and age

Among cattle, females were slightly more affected than males, although the difference was not statistically significant. Also, the condemned animals aged ≥2 years was significantly greater than those aged <2 years ($p < 0.001$). In contrast, the condemned sheep was significantly higher in males than in females ($p < 0.0001$) and in animals younger than 1 year (Table 2).

Causes for condemnations in slaughtered cattle and sheep

Post-mortem examination showed a varity abnomalities and diseases (Table 3, Figure 1). The identified conditions included:

pneumonia (Figure 2), emphysema (Figure 2), hemorrhagic lung (Figure 2), hepatitis, pulmonary hepatization, tuberculosis-compatible lesions, actinobacillosis, actinomycosis, hydatidosis (Figure 4), respiratory strongylosis, sarcoptic mange, lumpy skin disease (LSD), traumatic reticuloperitonitis (TRP), traumatic carcasses, abscess (Figure 2), rotten and hemorrhagic carcasses.

Condemnation of meat and offal

The total of 283 condemnation events comprised 279 organ condemnations and 4 carcass condemnations were condemned in the two animal species.

Table 2.
Number and percentage of condemnations of animal slaughtered based on sex and age

Cattle				Sheep			
Category	N	%	p-value	Category	N	%	p-value
Sex							
Male	41	44.08	0.25	Male	110	92.43	<0.0001
Female	52	55.91		Female	9	7.56	
Age							
< 2ans	28	30.10	<0.001	[6 months -1 years]	93	78.15	<0.001
≥ 2 ans	65	69.89		[1 years - 5 years]	16	13.44	
				> 5 years	10	8.40	

The count is at condemnation-animal level (N=93 for bovins/ N=119 for ovins).

Table 3.
Number and percentage of causes for condemnations in slaughtered cattle and sheep

Causes for condemnations	Bovins		Ovins	
	n/93	%	n/119	%
Pneumonia	10	10.75	46	38.65
Tuberculosis-compatible lesions	49	52.68	2	1.68
Hydatidosis	14	15.05	20	16.80
Abscess	11	11.82	19	15.96
Respiratory strongylosis	2	2.15	19	15.96
Emphysema	6	6.45	6	5.04
Hemorrhagic lung	0		8	6.72
Hepatitis	1	1.07	4	3.36
Hepatization of lung	0		4	3.36
Sarcoptic mange	0		4	3.36
Actinobacillosis and Actinomycosis	1	1.07	1	0.84
Traumatic carcasses	2	2.15	0	
Lumpy skin disease (LSD)	1	1.07	0	
Traumatic reticuloperitonitis (TRP)	1	1.07	0	
Rotten carcasses	1	1.07	0	
Bleeding carcasses	1	1.07	0	
p-value	< 0.0001		< 0.0001	

The count is at condemnation-animal level (N=93 for bovins/ N=119 for ovins).

Condemnations were significantly greater ($p = 0.005$) in cattle 165 (58.30%) than sheep 118 (41.69%). Among the 212 animals with at least one condemned organ or carcass, the lungs (n=186) were the most affected organ, followed by the hearts (n=49), livers (n=34), heads (n=6), stomachs (n=3) udders (n= 1) (Figure 5).

The lungs were the most condemned organ in both animal species. Heart and the liver condemnations were more common in cattle than in sheep, while carcass, stomach, and udders were condemned only in cattle. Head condemnation was more recorded in sheep than in cattle (Table 4).

The lung was seized for various reasons. In cattle,

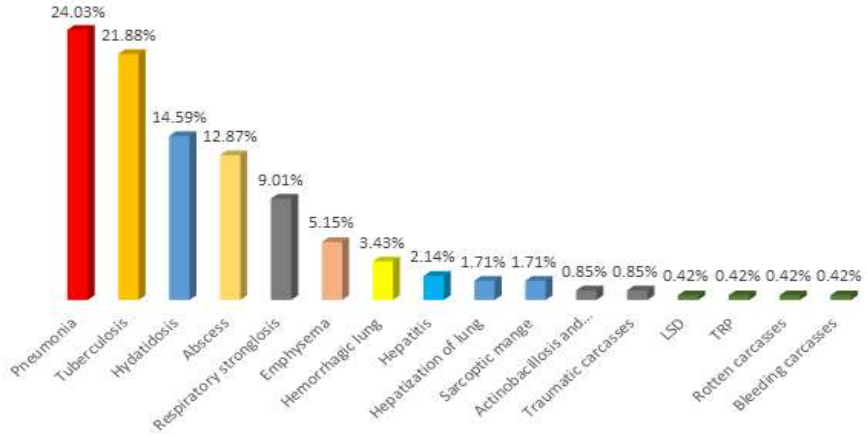


Figure 1.
Causes for condemnations in slaughtered animals.

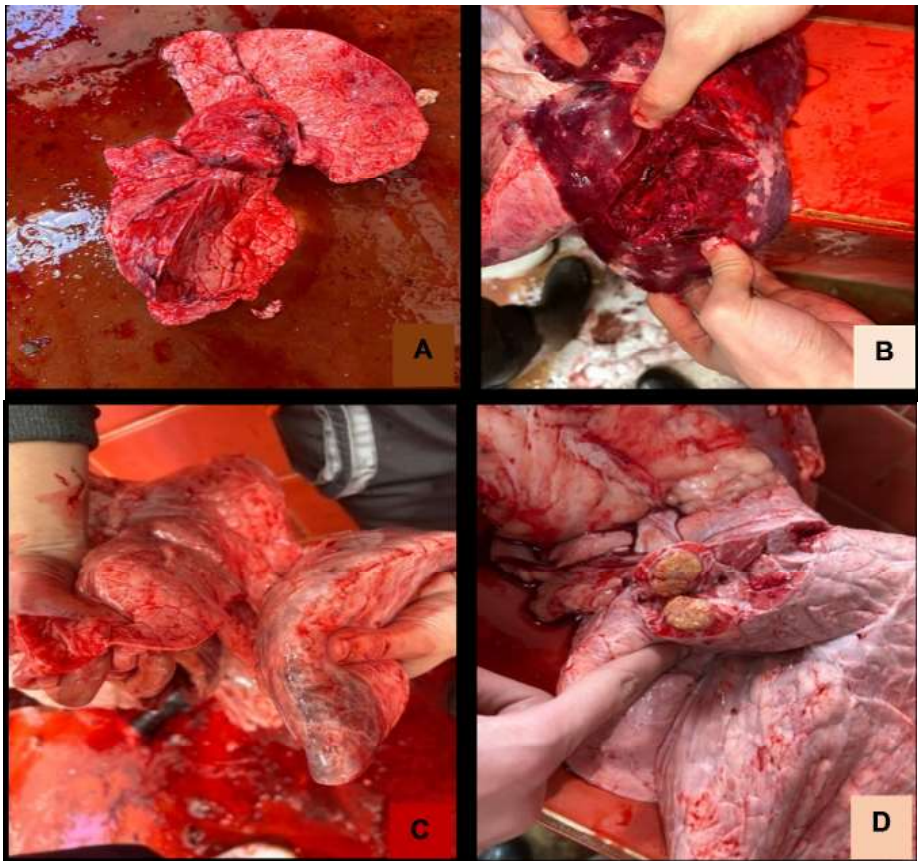


Figure 2.
Main pulmonary lesions encountered in the two animal species.
(A); Pneumonia, (B); Hemorrhagic lung, (C); Emphysema, (D); Abscess in the lung.

the main cause for lung condemnation was tuberculosis-compatible lesions and the least reason was TRP. In sheep, pneumonia was the most pathology recorded, while lower condemnation rates were recorded for tuberculosis-compatible lesions and strongylosis (Table 5).

In the case of liver condemnation, abscess for cattle and hydatidosis for sheep were the most frequently recorded causes, whereas TRP for cattle and abscess for sheep were the lowest recorded causes (Table 6).

Among the condemned hearts, tuberculosis-compatible lesions was the predominant cause in both species (Table 7).

Regarding head condemnation, four cases of sarcoptic mange, and one case of actinobacillosis were recorded in sheep. While in cattle, only one case of actinomycosis was recorded. The other organs were seized in cattle for tuberculosis, these are 3 cases for

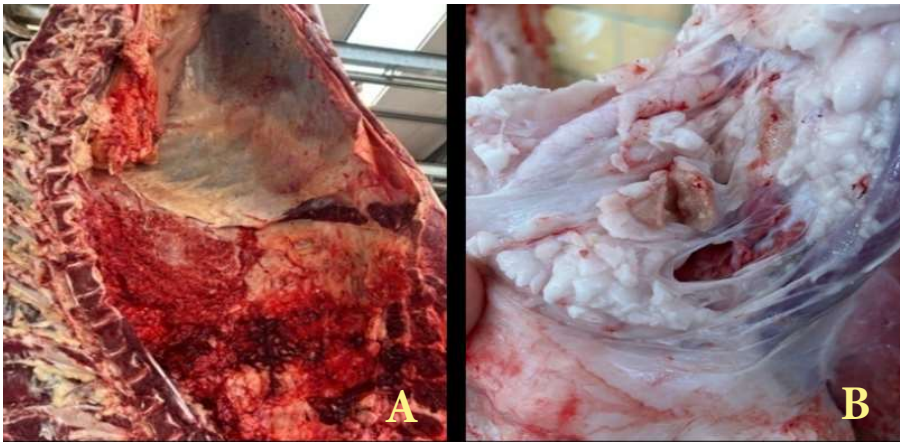


Figure 3.
Tuberculosis lesion.
(A); Tuberculosis in bovin carcass,(B); Tuberculous lymph node (black arrow).

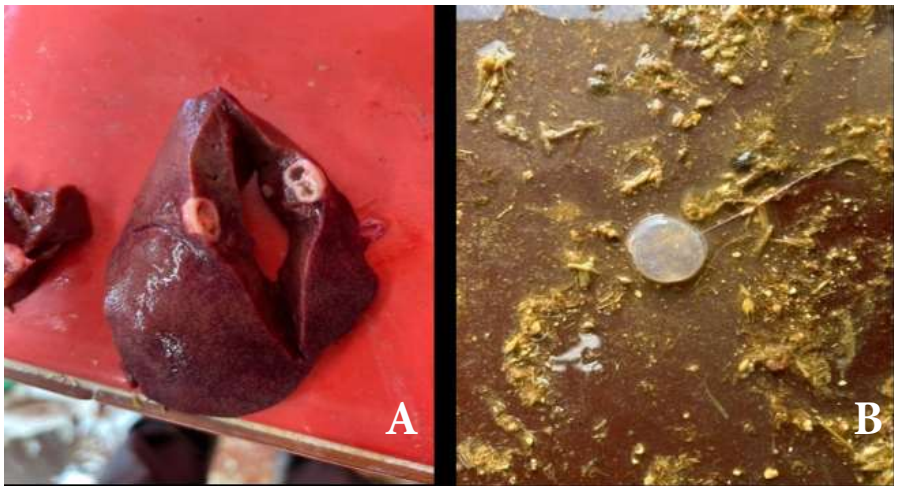
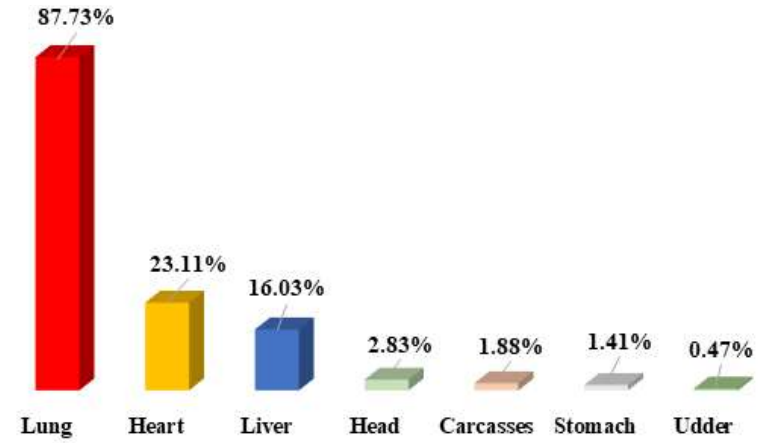


Figure 4.
Hydatid cyst in the liver.
(A); Dead and calicified cyst (B) Living cyst.



The count is at condemnation-animal level (N=212).

Figure 5.
Meat and organs seized from both animal species

the stomach and a single case for the udders. The four cases of carcasses condemnation were recorded for tuberculosis-compatible lesions associated with traumatic carcasses, abscess associated with traumatic carcasses, bleeding carcasses associated with LSD and finally rotten carcasses.

The partial and total organs and carcasses condemnations

Of the 283 condemned organs and carcasses, 162 (57.24%) cases underwent total condemnation, whereas 121 cases (42.76%) underwent partial condemnation. The type of condemnations according to

Table 4.
Organ-specific condemnation among cattle and sheep.

Seized meat and offal	Bovins		Ovins		p-value
	n/93	%	n /119	%	
Carcasses	4	4.30	0		/
Lung	85	91.39	101	84.87	0.24
Heart	46	49.46	3	2.52	<0.0001
Liver	25	26.88	9	7.56	0.0061
Head	1	1.07	5	4.20	0.22
Stomach	3	3.22	0		/
Udder	1	1.07	0		/

The count is at condemnation-animal level (N=93 for bovins/ N=119 for ovins).

Table 5.
The percentage of causes for lung condemnation..

Cattle (N=85)			Sheep (N=101)		
Causes for condemnations	n (%)	**p-value**	Causes for condemnations	n (%)	**p-value**
Tuberculosis-compatible lesions	48 (56.47)	<0.0001	Pneumonia	46 (45.54)	<0.0001
Hydatidosis	12 (14.11)		Abscess	19 (18.81)	
Pneumonia	10 (11.76)		Hydatidosis	14 (13.86)	
Abscess	6 (7.05)		Hemorrhagic lung	8 (7.92)	
Emphysema	6 (7.05)		Emphysema	6 (5.94)	
Respiratory strongylosis	2 (2.35)		Hepatization of lung	4 (3.96)	
TRP	1 (1.17)		Tuberculosis-compatible lesions	2 (1.98)	
			Respiratory strongylosis	2 (1.98)	

The count is at condemnation-organ level (N=85 for cattle/ N=101 for sheep).

Table 6.
The percentage of causes for liver condemnation.

Cattle (N=25)			Sheep (N=9)		
Causes for condemnations	n (%)	p-value	Causes for condemnations	n (%)	**p-value**
Abscess	9 (36)	0.02	Hydatidosis	5 (55.55)	0.26
Hydatidosis	8 (32)		Hepatitis	3 (33.33)	
Tuberculosis-compatible lesions	6 (24)		Abscess	1 (11.11)	
Hepatitis	1 (4)				
TRP	1 (4)				

The count is at condemnation-organ level (N=25 for cattle/ N=9 for sheep).

Table 7.
The percentage of causes for heart condemnation.

Cattle (N=46)			Sheep (N=3)		
Causes for condemnations	n (%)	p-value	Causes for condemnations	n (%)	**p-value**
Tuberculosis-compatible lesions	45 (97.82)	<0.0001	Tuberculosis-compatible lesions	2 (66.66)	-
TRP	1 (2.17)		Emphysema	1 (33.33)	

The count is at condemnation-organ level (N=46 for cattle/ N=3 for sheep).

the affected organs or carcasses are shown in figure 6.

Estimation of financial losses associated with organs and carcasses condemnation

For the economic loss, it was estimated according to the weights of the condemned organs and carcass, and their corresponding market pricees per kg of meat in the Blida region. Out of 1091 cattle and sheep carcasses inspected, 1200kg of carcasses and 485kg of organs were seized. Therefore, the total economic loss of both organs and carcasses was estimated at **30610.00** USD, as shown in Table 8.

Table 8.
Partial and total condemnations and estimation of economic losses.

Offal and Meat condemned	Total weight (kg)	Unit price in USD	Estimated loss in USD
Carcasses	1200	18.77	22524
Lung	215	18.02	3874.3
Heart	30	22.53	675.9
Liver	80	37.54	3003.2
Head	150	3.00	450
Stomach	5	15.02	75.1
Udder	**5**	1.50	7.5
Total			**30610.00**

Discussion

Many surveys conducted to investigate conditions and abnormalities have been performed in different countries abattoir [10] and numerous studies have investigated the causes and rates of carcass and organ condemnation in different countries, with considerable variation reported among studies. For example, a carcass condemnations rate of 0.37% was reported in cattle in Namibia Mbiri et al. [8]. In Iran, the rates of carcass and offal condemnations were 0.21% in cattle and camels and 0.02% in sheep and goats [9]. In Zambia, a condemnation rate of 0.55% was reported for cattle carcass and offal [7], whereas in Turkey, rates of 0.32% for carcasses and 2.33% for offals were reported in both sheeps and cattle [15]. In the USA, a cattle carcass condemnation rate of 2.01% was reported [16], while in Benin, the cattle carcass condemnation rate was 12.17% [17]. In Italy, condemnation rates of 2.77% in sheep and 23.87% in cattle were reported for both carcass and organs [18]. In Ethiopia, Ahmed et al. [2] reported an organ condemnation rate of 39.06% in cattle, while Sheferaw and Abdu [5] reported a rate of 69.2% for organ and carcass condemnations. In Algeria, a condemnation rate of 46.20% for sheep organ and carcass was reported [4] whereas in Egypte, rates of 8.7% in sheep and 87.7% in cattle were reported for both meat and offal [1]. These results must be carefully evaluated because the substantial difference among these studies may be explained by several factors like differences between the prevalence of diseases, the characteristics of the slaughterhouse, the data source, the study period, experimental design, geographical location, season, the meat inspection technique and the periode during which the studies were conducted [8, 10]. In the present study, parasitic lesions were more frequently associated with condemned organs in sheep than in cattle, which it's in agreement with other research works [2, 5, 9, 13, 14, 18, 19, 20]. This means that parasites may be responsible for considerable losses of meat, and especially offal, in sheep, more so than other diseases [11]. This could be mainly due to the presence of stray dogs and cats which can excrete parasites and contaminate plants and food intended for livestock [11]. This possible explanation or hypothesis was not evaluated in the current study, but stray dogs are frequently involved in environmentally transmitted diseases, particularly soil- and water-borne parasites, due to uncontrolled defecation and opportunistic behavior [21].

In the present study, sheep were more infested with parasites than cattle. This can be explained by the fact that sheep are raised in groups in extensive

farms in close and constant contact with stray dogs and cats [10, 22]. In the present study, the most frequent reason for condemnation for cattle was tuberculosis-compatible lesions 52.68%, consistent with the active circulation of *Mycobacterium* in algerian herds, particularly in old animals. Nevertheless, the high prevalence is explained by airborne transmission between animals, overcrowding of animals, the prolonged persistence of the bacillus in the environment, as well as by gaps in screening and eradication strategies [23]. In the present study, we acknowledg the lack of laboratory confirmation for these lesions but *M. bovis* infections account for a high amount of granulomatous lesions detected in Algerian slaughter cattle during standard meat inspection at Algiers and Blida abattoir [24]. Indeed, Sahraoui et al. [24] reported that from gross visible granulomatous lesions isolated from 100 cattle in the abattoirs of Algiers and Blida, *M. bovis* was isolated from 88 of them. The economic importance and public health significance of this zoonotic disease have been established in many countries [17]. Several previous studies have similarly identified tuberculosis-compatible lesions as the principal cause of condemnation [5, 7, 13, 17]. The third causes for condemnation for the both animal species was abscess. This corroborates with observations made by many authors [4, 7, 19, 20]. Abscess formation may result from infections involving a variety of bacterial pathogens, including *Corynebacterium pseudotuberculosis-compatible lesions, Staphylococcus aureus, Streptococcus spp*. These infectious agents can be transmitte to animals by breeding equipment [25]. Pneumonia have also been recorded in the present study, some authors have previously reported similar results [2, 4, 14, 16, 19, 20]. Others respiratory pathologies, including emphysema, hemorrhagic lung, hepatization, were also observed in the present study. The interlobular structural organization of the lungs in ruminants (lack of collateral ventilation), especially in cattle, could makes these animals more susceptible to respiratory diseases [26]. Some factors were reported in the literature such as poor ventilation in the sheepfolds, overcrowding in the livestock building, exhaustion during the dry season due to long treks in search of pasture and water, climate change, exposure to dust and parasitism may also play a role in the emergence of these respiratory diseases [4, 19, 26]. Because of these factors, lungs are may be the most condemned organ in the abattoirs [25]. This is observed in the present study and corroborates with various investigations in different countries [4, 9, 17, 25, 27]. However, others investigations [2, 5, 19, 20] have identified the liver as the most condemned organ. According to Grist [28], the liver and lung were highly infested and more susceptible than other organs because they are highly vascularized and function as a filter organ, potentially making them frequent sites for the localization of infectious agents. In the present study, lung condemnation in cattle was frequently associated with bovine tuberculosis-compatible lesions, which is consistent with observation reported by García-Díez et al. [10]. The thoracic cavity is known to be the main location of bovine tuberculosis-compatible lesions [10]. Similarly, inflammation of lung tissue and certain parasitic pathologies such as hydatidosis have also been considered as the most frequently encountered causes of lung condemnation [5, 10, 19, 20, 27]. Hydatidosis, the major parasitosis found in the present study, was reported to be the principal pattern of liver condemnation [19]. Condemnation for hepatitis and abscesses is expected because parasitic infestation can cause degeneration of liver tissue [10]. Heart condemnation in the present study was mainly associated with tuberculosis-compatible lesions and emphysema, while a very low percentage was associated with TRP. García-Díez et al. [10] reported that the heart damage was usually secondary to respiratory problems. The economic losses incurred in abattoir survey were **30610.00** USD according to the conversion rate used in the study. This economic loss was higher than those reported in some previous studies [8, 20, 26]. Differences between studies may be explained by variation in study plan, slaughterhouse output, price of meat and offal among countries, prevalence of different pathologies, rejection rate of organs [19, 20], currency conversion and year of estimation. However, no matter how significant these losses are, they always have a negative effect on meat production, abattoir income and the economy in general [8].

Conclusion

In conclusion, data collected at the two slaughterhouses of the Blida region made it possible to identify the main diseases affecting cattle and sheep. Among cattle, tuberculosis-compatible lesions is the most common, followed by hydatid cysts, abscesses, and pneumonia. In sheep, pneumonia predominates, followed by hydatid cysts, abscesses, and respiratory strongyles. These results highlight the need to step up upstream measures to prevent the occurrence of these conditions.

Limitations

The importance of the present study reveals that it is among the very rare abattoir survey study in the north of Algeria specially the region on Blida region.

However, several limitations should be considered when interpreting the findings. First, the limited number of visits per week (only 4 days) and the relatively short sampling period limits the ability of the study to show long-term trends and the rare events or pathogen spikes might be missed. Second, the study was conducted at only two abattoirs and the findings may not be representetive of all slaughterhouses in northern Algeria or the whole country, because each region may have distinct farming, hygiene, or processing conditions. Another important limitation is the possible effect of seasonal variation, beacause the present study was conducted during a single season which can missed high or low risk periods throughout the year, the weather and temperature change across the year which can affect microbial growth and animal health. In addition, study population may not perfectly represent the wider livestock population in Algeria.

Materials and Methods

Declaration of Generative AI and AI-assisted technologies in the writing process

There is no generative AI and AI-assisted technologies used in the writing process.

Ethical approval

This study involved routine ante-mortem and post-mortem inspection of animals slaughtered for commercial purposes and did not involve experimental procedures or additional interventions on animals.

Study area

This study was conducted from January to April 2025 in two slaughterhouses, Zaouia et Boufarik, located in Blida region of northern Algeria. Blida city is situated about 45 km southwest of the capital of Algiera, at an altitude of 190 m above sea level. The region has Mediterranean climate, with an average annual temperature of 17.9 °C and an average annual rainfall of 791 mm. The study area broadly fall within agricultural zone with significant production, farm animals are also diversified (cattle, sheep and goats).

Study population

The sample size for this study was determined based on the actual throughput and availability of animals brought to the slaughterhouse during the sampling period. Due to the lack of comprehensive pre-existing data on the local livestock population and to reflect real sample size, we chose to include all slaughtered animals that were accessible. This convenience sampling approach maximized the number of subjects included, given the facility's logistical and seasonal constraints. A total of 1091 animals, comprising 805 sheep and 286 cattle, intended for local consumption were included in the study. Animals were selected, with every fifth cattle and every tenth sheep, selected for examination. The selected animals were identified according to age and sex during ante mortem inspection. Cattle were classified into two age groups: < 2 years and ≥2 years, while sheep were classified into three groups: [6 months -1year], [1year- 5 years], ≥ 5 years. The animals' ages were provided by the breeders and, when possible, verified by examination of the animals' teeth. The cattle included local breeds and crossbreeds. Slaughter animals were transported to the abattoirs by road on approved transport vehicles and they were brought for slaughter from different districts around Blida region. It should be noted, The sum of the percentages for some measures exceeds 100% because individual animals may have been associated with more than one condemnation or cause of condemnation.

Study design and data collection

A cross-sectional study was conducted to determine the causes of condemnation in cattle and sheep and to estimate economic lossse. In this study each week four days visit was made for the examination of slaughtered animals which correspond to the days of highest slaughter volumes occuring over the two-days weekend period (Friday, Saturday), and on the two livestock market days (Monday, Wednesday). The animals were selected bby systematic sampling techniques and on each visit day, about 50-55 sheep and 17-18 cattle were examined. Accordingly, a total of 805 sheep and 286 cattle were examined. The study was conducted using previously designed questionnaires. The questionnaire collects and provides information on the slaughtered animals (age, sex, species), the type of lesion, organ affected, and the weight of the different condemned organs.

Methods of inspection (examination)

Routine examination was carried out by the qualified veterinary hygiene inspector assistants. Decisions were made to confirm or cancel the slaughter of the animals. The technical methods used to achieve reliable results followed the logic below :

Antemortem inspection

Before slaughter, each animal was examined for its general condition, nutritional status and the presence of any abnormalities or clinical sign. This inspection is carried out according to the following steps:

- Presentation of a slaughter authorization certificate is mandatory for any animal entering the slaughterhouse. This documentation allowed the health officer to distinguish between sanitary and ordinary slaughter, with verification of the animal's identification and ear tag numbers, where available ;
- An initial remote examination allowed the health officer to identify certain pathologies ;
- If necessary, the veterinarian performed a detailed clinical examination on certain animals.

Decisions were taken according to the actions specified by FAO [22]. Sick or infected animals, as well as the overworked were isolated in the designated holding area when required, while, animals considered suitable for slaughter proceeded to the slaughter process.

Postmortem inspection

This inspection was conducted approximately one hour after slaughter and it was carried out according to the following steps:

- A general inspection : to obtain a first impression of the animal's health after slaughter.
- Inspection of the viscera and organs: to detect any internal abnormalities in appearance, shape, color, consistency of all organs, especially the liver, lung, heart, kidneys. The examination of the viscera followed a systematic procedure, as indicated in the table 10. Pathological lesions were differentiated and the following judgment were used:
- Accepted for humain consumption : Meat and organs showing no disease or contamination and are safe for human consumption.
- Partial Condemnation: Only a small, localized part or single organ is removed, while the healthy rest is kept.

Table 9.
Gross pathological criteria used for classification of lesions [23, 31].,

Pathological conditions	Diagnostic of specific criteria
Tuberculosis compatible lesions	-Visual and palpation of yellowish-white, encapsulated granulomas with caseous or gritty calcified centers in target organs like lungs and associated lymph nodes (including the bronchial and mediastinal) as well as retropharyngeal and mesenteric nodes.
Pneumonia	-Visual and palpation of areas of dark red or grey consolidation (characterized by firm, heavy, non-collapsed lung tissue), often in the cranioventral lobes or fibrinous pleurisy adhesions on the lung surface. - In the airways: Presence of catarrhal, purulent, or bloody exudate inside opened bronchi and the trachea.
Hydatidosis	-Visual and palpation of fluid-filled vesicular cysts of varying sizes embedded in the tissue or bulging on the organ surface predominantly in the lungs and liver. - On incision: A distinct external white germinal/laminated wall with clear hydatid fluid and sometimes gritty brood capsules inside.
Respiratory trongylosis	-Visual and palpation of small nodular, subpleural gritty foci (Mullerius) or generalized rubbery worm-filled large bronchi (Dictyocaulus). -In the airways: Slicing major bronchi and the trachea reveals slender, thread-like white adult nematodes mixed with mucus or focal chronic bronchitis.
Emphysema	-Visual appearance: Pale pink to grayish-yellow, overinflated, bulbous lung lobes with visible subpleural air bubbles or pearl-like bullae. - On Palpation: Puffy texture with a characteristic spongy or crepitant feel.
Hemorrhagic lung	-Visual appearance: Focal, multifocal, or diffuse dark-red to black patches on the pleural surface and deep parenchyma. - On Palpation: Firm or spongy areas that ooze dark, unclotted or partially clotted blood upon incision.
Hepatitis	-Visual appearance: Alterations in normal reddish-brown color (pale, yellow-brown, or bright red), swelling, and presence of white/gray foci, fibrous scars, or migratory parasitic tracts. -Palpation and Incision: Increased organ consistency (firmness), rounded borders, and gritty or caseous textures upon cutting into portal lymph nodes or hepatic parenchyma.
Hepatization of lung	-Visual appearance: Lungs resemble liver tissue (heavy, solid, and airless). Divided into red hepatization (dark red, hyperemic, firm) and gray hepatization (grayish or pale yellow, dry, highly dense) stages typical of fibrinous or bacterial pneumonia. Palpation and Incision: Firm, heavy, and non-crepitant consistency; sinking in water. Cut surfaces display a dry, granular appearance without bubbling air.
Sarcoptic mange	-Thickened, wrinkled, and hyperkeratotic skin with crusts, papules, and large folds, predominantly starting around the head, neck, and shoulders.
Actinobacillosis	Firm, hard, nodular granulomatous swellings and suspected cases of abscesses containing thick, sticky, grayish-white or greenish-yellow pus. Primarily affects soft tissues, especially the tongue, lips, and associated head lymph nodes (infrequent in sheep)
Actinomycosis	Gritty pus or tissue scrapings show characteristic hard sulfur granules Involves hard bony tissues, characteristically the mandible or maxilla of cattle.
Traumatic carcasses	Visual assessment of asymmetrical tissue damage, hemorrhage in musculature, fractured bones, and torn or lacerated soft tissues.
lumpy skin disease	Subcutaneous nodules clearly visible after skinning; characteristic nodules and ulcers found across the mucous membranes of the digestive tract, trachea, and lungs.
Traumatic Reticulo-Peritonitis (TRP)	-Presence of metallic or sharp foreign objects, such as nails or wire, penetrating the reticular wall. -Localized peritonitis, fibrinous or purulent adhesions between the reticulum, diaphragm, and adjacent organs (liver or spleen).
	-Abnormal, foul, putrid, or sour odors emanating from the meat, body cavities, and musculature. -Discoloration of the flesh (greening, darkening, or hyperemic blood pooling), loss of muscle tone, and gas accumulation or bloating in uneviscerated parts.
Bleeding carcasses	- Presence of marked blood congestion in subcutaneous, axillary, and intercostal blood vessels are full of blood and congested. - Presence of an abnormal dark, dull, or wet meat appearance (instead of a bright, well-set, dry surface).

All lesions were classified on the basis of gross post-mortem examination. The laboratory confirmation is not done.

Table 10.
Visceral inspection technique [30].

Organ/System	Steps of examination
Heart and pericardium	-Visual examination of the pericardial sac (parietal pericardium); -Visual observation and palpation of the heart; -Incision of the pericardium and observation of the fluid; -Longitudinal incision to open the ventricles and penetrate the interventricular septum
Lungs, trachea, esophagus	-Visual examination of the lungs: appearance, volume, color, etc.; - Palpation of all lobes, from the hilum to the periphery; - Incision and examination of the tracheobronchial, mediastinal, and apical lymph nodes; - Incision of the trachea and bronchi and of each lung and examination of the pulmonary parenchyma; - Visual examination and palpation of the esophagus.
Liver	- Visual examination ; - Palpation of both surfaces ; - Palpation and incision of the hepatic lymph nodes ; - Two incisions on the visceral surface : • A long and shallow incision between the right and left lobes ; • A short and deep incision involving the caudate lobe.
Spleen	-Visual examination and palpation ; - Incision when necessary.
Kidney	-Visual examination and palpation ; - Incision along a sagittal plane ; - Incision and examination of the renal lymph nodes.
Digestive tract	- Visual examination : rumen-reticulum junction, mesentery, omentum, intestinal wall ; - Palpation, incision when necessary of the gastric and mesenteric lymph nodes.
Uro-genital tract	-Visual examination of the genital organs and uterine contents; - Palpation and incision when necessary (without contaminating the carcass); - Incision of the scrotal nodes in males.
Head, tongue	-Visual examination of the external surfaces of the head ; -Incision of the mandibular (submandibular), parotid, and lateral and medial retropharyngeal lymph nodes. - Visual examination and dorsoventral palpation of the tongue.

The inspection procedure was adopted from the order of 17 March 1992. Official Journal of the French Republic;1992.

-Total Condemnation: The entire carcass and all organs are destroyed due to widespread disease or toxic risks [29].

Estimation of economic losses attributed to organ and/or carcass condemnations

The estimation of economic losses was calculated according to the weight of condemned organs and meat and their average local market prices (per kg) during the study period. The unit prices were obtained from Commercial Services Directorate for the Blida Region– April 2025.These prices represented the butcher prices and were applied to all animal species. During the study period, the prices for the liver, lung,heart,beef,head, stomach, udder was 37.54 USD, 18.02 USD, 22.53 USD, 18.77 USD, 3.00 USD, 15.02 USD and 1.5 USD respectively. The total economic loss was calculated using the following equation:

Total loss = (C * PC) + (Lu * PLu) +(H * PH) + (L * PL) + (He * PHe) + (S * PS) + (U * PU). Where in kg, C = Carcasses total condemned weights, Lu = lung total condemned weights, H = heart total condemned weights, L = liver total condemned weights, He = total condemned weights, S=Stomach total condemned weights, U = Udder total condemned weights Where in USD, PC = Unit price of carcasses, PLu = Price of lung, PH = Price of heart, PL = Price of liver, PHe = Price of head, PS = Price of stomach, PU = Price of udder.

Statistical analysis

For statistical analysis, software program Microsoft Excel 2010 and IBM SPSS Statistics Version 20 (© IBM Copyright, IBM Corporation and its licensors 1989.2011 IBM., USA) was used. The collected data were analyzed according to animal species (cattle and sheep), gender, age, and other relevant factors based on the number and percentage of animals slaughtered and recorded in the slaughterhouses. Confidence intervals (CIs) at a 95% confidence level were calculated using SPSS. The Chi-square test of homogeneity was applied to compare the different factors. Statistical significance was set at $p \leq 0.05$.

Data-availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

A.D Concept and design of the study, recording data, drafting of the manuscript, data analysis, interpretation of the results, and prepared the final draft of the manuscript. S.Z performed the statistical analysis, revising the manuscript and final approval of the paper following critical editing. All authors read and approved the final manuscript for publication.

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Competing Interests

The authors declare that there is no conflict of interest.

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Case Report

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Clinical and radiographic findings of Monteggia fracture in a one-year-old-male mule

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ABSTRACT

The elbow joint is between the distal humerus and proximal radius and ulna. In uncommon cases, severe traumatic injury to the elbow may result in concomitant fracture and luxation or subluxation of its structure. The Monteggia fracture is defined as dislocation of radial head associated with proximal ulnar fracture. This injury is rare in the horse and to the authors' knowledge, no previous reports in mule. Concurrent fractures of the ulna and radius can be particularly challenging. Because of the extensive soft tissue and articular cartilage injuries, cases with Monteggia fracture have an unfavorable prognosis. Although, successful surgical management has been documented in some cases. Even though the successful reports of repair of Monteggia fracture, these cases are as pasture-sound animals. This report describes a type I Monteggia fracture in a one-year-old male mule. Clinical examination revealed severe swelling, pain, crepitation and non–weight-bearing lameness. Radiographic evaluation confirmed the Monteggia fracture. Given the little information available regarding Monteggia fracture in equine, a better understanding of treatment of this injury is warranted.

Keywords

Elbow joint, Equine, Luxation, Monteggia fracture, Young mule, Radius, Ulna

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Abbreviations

No abbreviations

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Introduction

The humeroradial (elbow) joint is between the distal humerus and proximal radius and ulna. The stability of the elbow joint is supported by collateral ligaments, while its joint capsule attaches to the margins of the articular surfaces of the humerus, radius, and ulna bones [1]. In equines, elbow lameness is commonly associated with direct trauma, falls, or even collisions with objects or other animal. In uncommon cases, severe trauma may lead to concomitant luxation of the humeroradial joint and fracture of the proximal part of the ulna, a condition known as a Monteggia fracture [1, 2, 3]. This injury was first described in 1814 by Giovanni Battista Monteggia [4]. Originally Monteggia is rare condition in equines [5, 6, 7, 8] and to the authors' knowledge, this condition has not previously been reported in mule. The present case report describes clinical and radiographic findings of a type I Monteggia fracture in a one-year-old male mule.

Case Presentation

Ethical Considerations

This manuscript reports a clinical case managed as part of routine veterinary care. All diagnostic procedures were performed in accordance with accepted standards of veterinary clinical practice and relevant animal welfare guidelines.

History and clinical finding

A one-year-old 180 kg male mule was referred to the Veterinary Clinic of Ferdowsi University of Mashhad with a history of a sudden acute lameness of the left forelimb.

The mule became trapped by a rope wrapped around a tree and sustained trauma while attempting to escape. On clinical examination a non-weight bearing lameness with a dropped elbow posture of the affected limb was observed. The elbow joint and antebrachium were severely swelled (Figure 1). Palpation of the affected region elicited severe pain, crepitation and bone displacement. Based on the clinical findings, a fracture or dislocation was suspected.

Radiographic Findings and Interpretation

Mediolateral and craniocaudal radiographic views of the left elbow were obtained (X-ray machine (Soyeet SY-HF-110, Soyee Product, Inc., Kangnam-Ku, Seoul, Gyeonggi, 135–729, Korea, 2.5 mA s, 74 kV)). Radiographs revealed complete anterior luxation of the humeroradial joint associated with a comminuted fracture of the proximal ulna (Figure 2). These radiographic findings were consistent with a type I Monteggia fracture.

Treatment

Surgical correction was recommended and the available treatment options were described with the owner. However, the owner declined surgical intervention.

Results and Discussion

The elbow is a hinge (ginglymus) joint. Articulation of the radial head with the semi-lunar notch of the ulna formed the humeral capitulum. During extension of the elbow joint, the anconeal process of the proximal part of the ulna rests into the olecranon fossa of the humerus. This provides an interlocking mechanism that stabilizes the elbow joint. The radius and ulna are closely associated with each other through four important interosseous ligaments including the cranial, caudal, medial and lateral collateral ligament. These ligaments provide broad-based attachments that distribute the high tensile forces generated during flexion and extension of the elbow joint. Because of these anatomical characteristics, the injuries of the elbow joint are rare, and often involve external trauma or association with a catastrophic event [1].

Monteggia fractures are classified into four types based on the direction of the radial head dislocation and the associated ulnar fracture pattern. Type I consists of anterior radial head dislocation with an ulnar fracture. Type II involves posterior or posterolateral radial head dislocation with an ulnar fracture. Type III is characterized by lateral or anterolateral radial head dislocation with an ulnar fracture. Type IV consists of anterior radial head dislocation with a fracture of both radius and ulna [4, 9].

The pathophysiology of Monteggia fracture in animals has not yet fully understood. The proposed mechanism involves the application of force or trauma to the caudal aspect of the antebrachium while the limb is fully extended and weightbearing [5]. Differential diagnoses for this condition include fractures of the humerus, radius or olecranon, as well as brachial plexus paralysis, all of which can be differentiated radiographically.

Only a limited number of reports describing surgical correction of Monteggia fractures in horses are available in the veterinary literature [5-8]. Management of Monteggia fractures can be challenging in horses because it needs extensive excision and reduction of the radius is difficult. Complete reduction of the radius requires traction supplemented by neuromuscular blockade and the use of a mechanical distractor, followed by the ulnar fracture stabilization using a caudally positioned dynamic compression plate

(DCP) engaging the radius, with or without additional plate applied to the lateral or craniolateral aspect of the radius [3, 5-7, 10]. However, in addition to ulnar fracture repair, determination of the lateromedial stability of the elbow joint and interdigitation of the three bones is necessary to provide sufficient stability. Although partial damage to one collateral ligament may be amenable to repair, complete disruption of one ligament or disruption of both ligaments substantially worsens the prognosis. In such cases, euthanasia may be warranted [8].

To the authors' knowledge, only five cases of Monteggia fracture in horses have been reported previously. Levine et al. described successful surgical treatment of a Monteggia fracture in a three-year-old Arabian horse using a dynamic compression plate, and reported that this filly was free of any apparent lameness [7]. Trostle et al. reported successful surgical treatment of a 27 month-old Rocky Mountain horse using a DCP. In that case, wound dehiscence and surgical site swelling, lameness and infection developed ten days after surgery. This horse became sound several months after management of these complications, although radiographic examination revealed bony proliferation and dystrophic mineralization along the proximolateral aspect of the ulna and distolateral aspect of the humerus associated with collateral ligament trauma [5]. Jalim et al. reported surgical treatment of a Monteggia fracture in a four-month-old, Anglo-Arab filly using a DCP plate; however, mild flexural deformity and advanced secondary degenerative joint disease subsequently occurred [6]. Janicek reported a fourteen-year-old Tennessee Walking gelding horse with Monteggia fracture that was finally euthanized because of the poor prognosis associated with maintaining elbow joint congruity and stability [8]. In another case with Monteggia fracture, the horse euthanized because of the concurrent radius fracture and the small size of the proximal radial fragment, which precluded adequate fixation [10]. In the present case, different aspects of this condition, including its treatment options, outcomes, prognosis and animal welfare, were fully discussed with the owner; however, the surgical treatment was refused by the owner.

It confirms that one of the possible consequences of elbow injuries, especially after fractures and soft tissue injuries is osteoarthritis [2]. The evidence of this condition may be affected by the degree of articular disruption due to severity of initial trauma [1]. Consequently, degenerative joint disease of elbow joint is generally associated with a guarded prognosis. These patients are as pasture-sound animals.

Recovery from anesthesia presents another challenge; so, it should be assisted for prevention of implant failure, humeroradial luxation or refracture of the ulna [8].

Surgical site infection is another complication of horses undergoing surgical repair of monteggia fractures and prevention of this condition is necessary [5].

A well-recognized complication of olecranon fracture repair is acquired flexural deformity. Therefore, timely removal of the initial instigating factor including orthopedic implants and prevention of permanent joint capsule contracture are necessary [1].

Athough the successful reports of repair of Monteggia fracture in horses have been reported, the prognosis of this condition for athletic performance remains guarded. The extensive destruction of soft tissue, articular cartilage and joint capsule, types of bone fracture, osteoarthritis and limb deformity often preclude a successful outcome and surgical management preserve the horse for pasture or breeding soundness [5, 6].

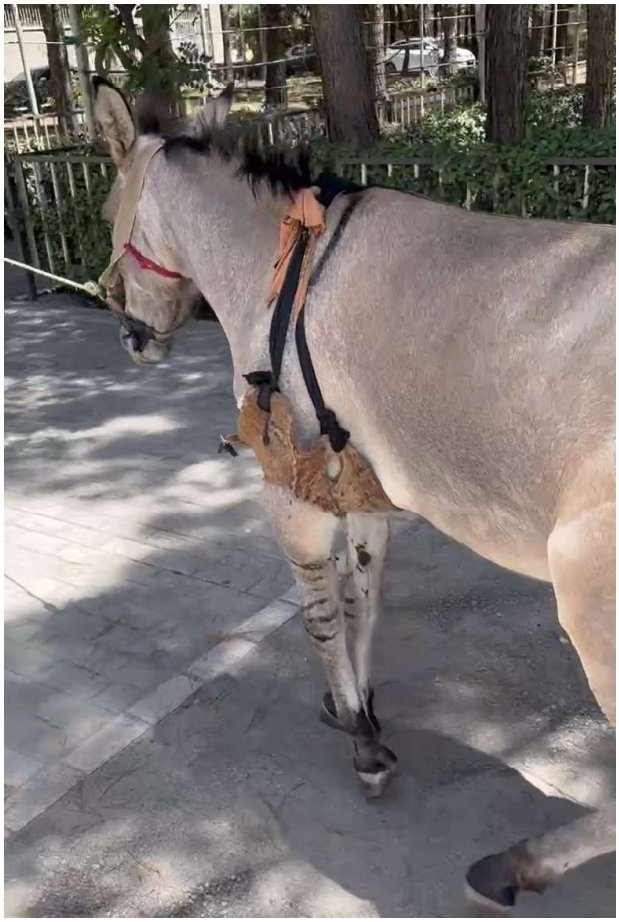

Figure 1.
Left forelimb non-weight bearing lameness with severe swelling of elbow region.

Authors' Contributions

All authors have participated sufficiently in this study reported in the manuscript and approved the manuscript and this submission.

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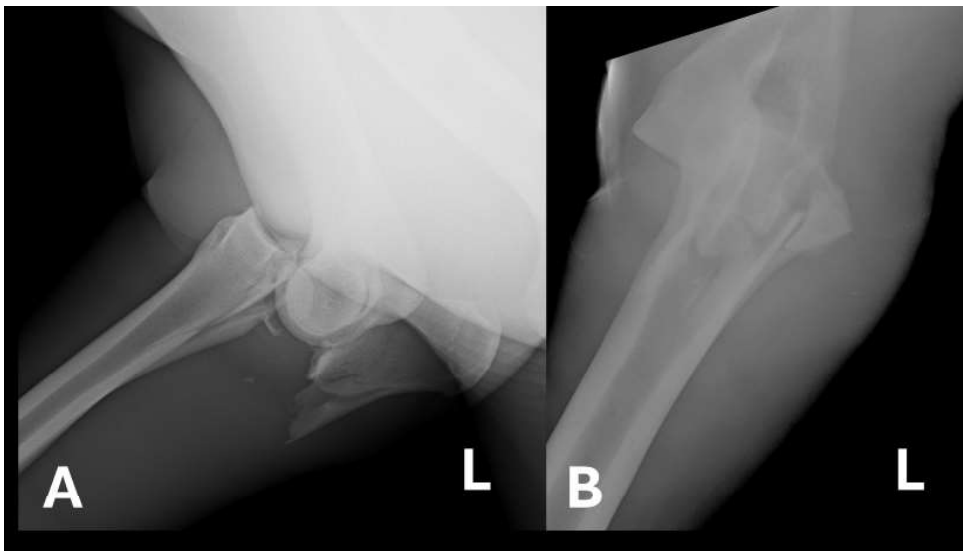

Figure 2.
Radiographic images obtained in mediolateral (A) and craniocaudal (B) projections of the left forelimb. The images demonstrate a Type I Monteggia fracture, characterized by a spiral and comminuted fractures of the proximal ulna associated with proximal and cranial luxation of the radial head, along with caudal displacement of the proximal ulnar fragment. Luxation of the humeroradial joint is evident, with the humeral condyle positioned caudal to the proximal radial metaphysis.

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Competing Interests

The authors declare that there is no conflict of interest.

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Abstract in Persian

باکتری *Kocuria rhizophila* عامل عفونی ماهی قزل آلای رنگین کمان (*Oncorhyn-chus mykiss Walbaum,1792*) در ایران

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چکیده

با شیوع طبیعی یک بیماری در دو مزرعه پرورش ماهی قزل آلای رنگین کمان واقع در شمال ایران که به صورت حاد بروز کرد، علائم بالینی از جمله؛ تیرگی چشم، پوسته پوسته شدن و نکروز پوست، زخم های سطحی تا عمیق در پشت و پهلوها، از بین رفتن کامل بافت دمی، اگزوفتالمی خفیف، ملانیزاسیون پوست، خونریزی و رنگ پریدگی کبد، مخاط فراوان در حفره شکم، کلیه های رنگ پریده و عفونت ثبت شد. پس از تجزیه و تحلیل بیوشیمیایی باکتری، توالی یابی ژن انجام شد که منجر به شناسایی و ثبت سویه ای به نام *K. rhizophila TMU97910* در بانک ژن *NCBI* شد. در پی مشخص شدن هویت باکتری، تست های تحمل به شوری و آنتی بیوگرام نیز صورت پذیرفت. نتایج نشان داد که سویه هم راستا با خصوصیات درون گونه‌ای خود، مقاومت کاملی در برابر شوری از خود نشان می‌دهد که به وی را قادر به زیستن در محیط های با شوری صفر تا اشباع از نمک می سازد.. این سویه درحالیکه به فسفومایسین مقاومت نشان داد، به فلورفنیکول، داکسی سیکلین، اکسی تتراسایکلین، انروفلوکساسین، سولفامتوکسازول/تری متوپریم، اریترومایسین، جنتامایسین و کانامایسین حساس بود. برای بررسی رفتار این باکتری در ماهی قزل آلای رنگین کمان،تست بیماری زایی، LD_{50} و آنالیز هیستوپاتولوژی در سه تکرار انجام شد. علائم بیماری ایجاد شده در ماهی قزل آلا به سان بروز آن در مزرعه،ثابت بود. بررسی هیستوپاتولوژیک کبد، کلیه، طحال و آبشش آسیب شدید ناشی از این سویه از جمله نکروز، خونریزی، دژنراسیون و زخم های بافتی را نشان داد. پس از آن، از بافت ماهیان بیمار شده کشت مجدد باکتری تهیه شد و نتایج منفی برای تشخیص سایر عوامل بیماری زا، تایید کرد که *K. rhizophila* به تنهایی مسئول این بیماری می باشد.

واژگان کلیدی

کوکیوریا ریزوفیلا، ماهی قزل آلا، بیماری زایی، بافت شناسی، تست آنتی بیوگرام

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تهیه و ارزیابی ایمونولوژیک پلاسما هیپرایمن سگ علیه پاروویروس سگ: یک استراتژی ایمونوتراپی غیرفعال

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چکیده

هدف از انجام این مطالعه تهیه پلاسمای هیپرایمن ضد پاروویروس سگ سانان با واکسیناسیون مکرر سگ و ارزیابی قدرت خنثی‌سازی و واکنش آن با آنتی ژن های ویروسی در مقایسه با پلاسمای گروه های سالم و بیمار بود. چهار قلاده سگ سالم چهار بار با فاصله دو هفته با واکسن زنده تضعیف‌شده CPV واکسینه شدند. پلاسمای هیپرایمن آنها جمع‌آوری و IgG آن با روش کروماتوگرافی تعویض یونی خالص شد. ویروس پارو سگ سانان از نمونه مدفوع سگ های بیمار ارجاعی به درمانگاه جداسازی شد و در سلول‌های MDCK تکثیر یافت و وجود آن با روش PCR بر روی ژن VP2 تأیید شد. تیتر آنتی‌بادی خنثی‌کننده با آزمون خنثی‌سازی ویروس، عیار آنتی‌بادی اختصاصی با الایزای غیرمستقیم و ایمونوژنسیته پروتئین‌های ساختاری) و غیرساختاری با روش وسترن بلات تعیین شد. پلاسمای هیپرایمن بالاترین عیار خنثی‌سازی (64 ± 160) را در مقایسه با سگ‌های سالم (12 ± 26) و مبتلا (2/5 ± 4/5) نشان داد. ($p \leq 0.01$) مقدار جذب نوری در الایزا برای پلاسما هیپرایمن، سالم و بیمار به ترتیب 11/0 ± 0/12..63/0 ± 0/50 و 0/02 ± 0/28 بود. ($p \leq 0.001$) وسترن‌بلات وجود آنتی بادی واکنش دهنده با VP2 (2/7 ± 0/66)، VP3 (3/5 ± 0/45) VP1 (14/3 ± 30/7) و NS1 (1/7 ± 0/68) را در گروه هیپرایمن نشان داد، در حالی که در سگ‌های مبتلا فقط میزان اندک آنتی بادی علیه VP2 وجود داشت. بر خلاف حیوانات بیمار، آنتی‌بادی علیه NS1 به شکل منحصر به فردی در پلاسمای هیپرایمن دیده شد. پلاسمای هیپرایمن سگ حاوی آنتی‌بادی‌های خنثی‌کننده با تیتر بالا و طیف گسترده علیه CPV است. این یافته ها از ارزیابی بالینی پلاسمای هیپرایمن به‌عنوان ایمونوتراپی اضطراری برای عفونت CPV حمایت می‌کند.

واژگان کلیدی

پاروویروس سگ، پلاسمای هیپرایمن، ایمونوتراپی غیرفعال، سگ

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اثرات تزریق داخل بطنی مغزی نایسین بر رفتار تغذیه‌ای، دمای بدن و پارامترهای سرم در جوجه‌های گوشتی ملتهب شده با LPS

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چکیده

لیپوپلی‌ساکارید (LPS) منجر به اختلال در عملکرد تولید، کاهش مصرف خوراک، تغییر دمای بدن و تغییرات مضر در برخی از پارامترهای سرم و بیماری در جوجه‌های گوشتی می‌شود. باکتریوسین‌هایی مانند نیسین به عنوان عوامل درمانی امیدوارکننده برای پیشگیری، کنترل و درمان بیماری‌های طیور در نظر گرفته می‌شوند. این مطالعه شامل آزمایشی با استفاده از ۳۰ جوجه گوشتی نر برای ارزیابی اثرات اصلی نیسین بر مصرف خوراک و آب، دمای بدن و فاکتورهای سرم تحت استرس بود. در این آزمایش، نیسین (۱ میکروگرم) و LPS (۲۵ نانوگرم) به صورت داخل بطنی تزریق شدند و درمان‌های ترکیبی نیسین و LPS نیز در نظر گرفته شد. مصرف خوراک، مصرف آب و دمای بدن در همه گروه‌ها اندازه‌گیری شد. در پایان آزمایش، خون از جوجه‌ها برای ارزیابی فاکتورهای سرم جمع‌آوری شد. تجویز داخل بطنی نیسین به طور قابل توجهی مصرف خوراک را افزایش داد، در حالی که تجویز LPS به طور قابل توجهی آن را کاهش داد. در درمان‌های ترکیبی، نیسین توانست تا حدی اثرات نامطلوب LPS بر مصرف خوراک را کاهش دهد. هم نایسین و هم LPS مصرف آب را در جوجه‌های گوشتی کاهش دادند و درمان ترکیبی باعث کاهش بیشتری در مصرف آب نسبت به هر تزریق به تنهایی شد. LPS در ابتدا دمای بدن را قبل از القای هیپرترمی کاهش داد، در حالی که نایسین به تنهایی هیچ تاثیری بر تنظیم دما نداشت، اما در گروه‌های ترکیبی، تا حدودی اختلالات دمایی ناشی از LPS را کاهش داد. LPS همچنین باعث کاهش قابل توجه آلبومین و افزایش قابل توجه AST و ALT شد، در حالی که نایسین AST و ALT را کاهش داد اما هیچ تاثیری بر آلبومین نداشت. نتایج نشان می‌دهد که نایسین می‌تواند به عنوان یک افزودنی مفید و ایمن در خوراک طیور استفاده شود.

واژگان کلیدی

نایسین، لیپوپلی ساکارید، رفتار تغذیه‌ای، دما، پارامترهای سرم، جوجه‌های گوشتی

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بررسی ریخت‌شناسی و مولکولی دو گونه تنیا در برخی از سگ‌سانان وحشی در شمال ایران

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چکیده

سگ‌سانان وحشی نقش مهمی در انتشار تخم کرم‌های انگلی دارند که می‌تواند به عنوان منبع بالقوه عفونت در انسان و حیوانات عمل کند. از این رو، پایش فون کرم‌ها در چنین گونه‌هایی از نظر دامپزشکی و بهداشت عمومی از اهمیت بالایی برخوردار است. مطالعه مقطعی حاضر به منظور ارزیابی عفونت سستود و ارزیابی خصوصیات مولکولی و وضعیت فیلوژنتیکی تنیاهای بزرگ در سگ‌سانان وحشی در ایران انجام شد. برای این منظور، روده ۹۳ سگ‌سان وحشی، شامل ۶۹ شغال، ۲۲ روباه قرمز و دو گرگ، در شمال، شمال شرقی و شمال غربی ایران از نظر وجود کرم‌های نواری بررسی شد. علاوه بر این، هویت برخی از کرم‌های روده با استفاده از PCR و تعیین توالی DNA تأیید شد. بر اساس یافته‌های ما، ۵۴/۵ ٪ از روباه‌های قرمز و ۴۷/۸ ٪ از شغال‌ها آلوده بودند که در میان آنها عفونت‌های تکی با مزوسستوئیدس(*Mes-ocestoides*) بسیار شایع بود (۳۳/۳ ٪)، و پس از آن تنیا هیداتیژنا (*T. hydatigena*) با شیوع ۴/۳ ٪، دیپیلیدیوم کانینوم (*D. can-inum*) با شیوع ۲/۲ ٪، تنیا پلی آکانتا (*T.polyacantha*) با شیوع ۱/۱ ٪. همچنین، عفونت‌های مختلط در ۱۳/۶ ٪ از روباه‌ها (Mes-ocestoides + *T. polyacantha*) و ۵/۸ ٪ از شغال‌ها (*Mesocestoides* + *T. hydatigena*) مشاهده شد. به طور کلی، میزان عفونت در گوشتخواران وحشی 48/4 ٪ بود. بررسی مولکولی با پرایمرهای یونیورسال میتوکندری، قطعه ۴۵۰ جفت بازی از ژن cox1 را ایجاد کرد. همچنین توالی‌یابی ۱۰۰ ٪ و >۹۹ ٪ شباهت بین توالی‌های جدایه‌های تنیا هیداتیژنا (n = ۸) و تنیا پلی آکانتا (n = ۴) و توالی‌های مرجع موجود در بانک جهانی ژن را نشان داد. این سومین گزارش و اولین مورد تایید شده مولکولی تنیا پلی‌آکانتا در روباه‌ها در ایران است. این یافته‌ها بر اهمیت پایش عفونت‌های سستودی در سگ‌سانان وحشی با هدف‌های اپیدمیولوژیک و بهداشت عمومی تأکید می‌کنند.

واژگان کلیدی

سستود، گوشتخواران وحشی، تنیا پلی‌آکانتا، تنیا هیداتیژنا، ایران

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مقایسه ایمنی‌زایی نسبت‌های مختلف آدجوانت کیتوزان در واکسن تضعیف‌شده نئوسپورا کنینوم در موش‌های BALB/c

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چکیده

نئوسپوروزیس یک بیماری عفونی عصبی– عضلانی در سگ‌ها است که توسط تک‌یاخته نئوسپورا کنینوم ایجاد می‌شود. این بیماری به‌طور گسترده در جهان پراکنده است؛ سگ‌ها و سگ‌سانان میزبان نهایی هستند و میزبان‌های واسطه متنوعی نیز وجود دارند. بنابراین، هدف این مطالعه مقایسه ایمنی‌زایی نسبت‌های مختلف آدجوانت کیتوزان در واکسن تضعیف‌شده نئوسپورا کنینوم در موش‌های BAL-B/c بود. بیست و هشت موش BALB/c به چهار گروه ۷تایی تقسیم شدند. گروه‌های ۱، ۲ و ۳ به‌صورت زیرجلدی با تاکیزوئیت‌های تضعیف‌شده نئوسپورا کانینوم سویه Nc-1 همراه با آدجوانت کیتوزان میکرونیزه شده به نسبت‌های ٪۱۰، ٪۵۰ و ٪۹۰ ایمن‌سازی شدند. گروه چهارم به عنوان کنترل در نظر گرفته شد. تمام گروه‌ها به مدت سه هفته روزانه تحت نظر قرار گرفتند. پس از نمونه‌برداری، تیتر آنتی‌بادی‌ها توسط ELISA اندازه‌گیری و ایمنی سلولی با سنجش میزان گاما اینترفرون با کیت تجاری ارزیابی شد. نتایج ELISA، بالاترین سطح آنتی‌بادی در گروه ایمن‌شده با واکسن تضعیف‌شده به همراه ٪۹۰ آدجوانت کیتوزان را نشان داد که اختلاف معنی‌داری نسبت به گروه کنترل و سایر گروه‌ها داشت ($p < 0.05$). در اندازه گیری گاما اینترفرون نیز، قوی‌ترین پاسخ ایمنی سلولی مربوط به گروه ٪۹۰ کیتوزان بود که نسبت به گروه کنترل و سایر گروه‌ها تفاوت معنی‌دار داشت ($p < 0.05$). بر اساس این نتایج و اثر قابل توجه آدجوانت کیتوزان بر تیتر آنتی‌بادی‌ها و ایمنی سلولی، استفاده از این آدجوانت در توسعه واکسن‌های نئوسپورا توصیه می‌شود.

واژگان کلیدی

.واکسن، نئوسپورا کنینوم، آدجوانت، کیتوزان

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یافته‌های بالینی و رادیوگرافی شکستگی مونتجیا در یک راس قاطر نر یک‌ساله

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چکیده

مفصل آرنج بین بخش پایینی استخوان بازو و بخش بالایی درشت‌نی و نازک‌نی است. در موارد غیر معمول، آسیب شدید وارد شده به مفصل آرنج منجر به رخداد هم‌زمان شکستگی، دررفتگی کامل یا ناکامل می‌شود. شکستگی مونتجیا به عنوان جابه‌جایی سر درشت‌نی همراه با شکستگی بالای نازک‌نی تعریف می‌شود. این شکستگی آسیب نادری است، به‌ندرت در اسب رخ می‌دهد و در قاطر گزارش نشده است. شکستگی هم‌زمان درشت‌نی و نازک‌نی به‌ویژه چالش‌برانگیز است. به‌دلیل جراحات وسیع بافت نرم و غضروف مفصلی، موارد درگیر با شکستگی مونتجیا پیش‌آگهی نامطلوبی دارند. به‌هر حال، بعضی گزارش‌ها مدیریت موفقیت‌آمیز این شرایط را ثبت کرده‌اند. علی‌رغم گزارش‌های موفق ترمیم شکستگی مونتجیا، این حیوانات برای گشت آزاد مناسب هستند. این مقاله شگستگی مونتجیا را در یک راس قاطر گزارش می‌کند. تورم شدید، درد، کریپتان و لنگش مرتبط با عدم وزن‌گیری مشاهده شد. ارزیابی رادیوگرافی، شکستگی مونتجیا را تایید کرد. اطلاعات اندکی در مورد شگستگی مونتجیا در اسب‌سانان وجود دارد و درک بهتر درمان این جراحت ضروری است.

واژگان کلیدی

مفصل آرنج، اسب، دررفتگی، شکستگی مونتجیا، قاطر جوان، درشت‌نی، نازک‌نی

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Author Index

Author index

Guide for authors

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.

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Manuscripts submitted to IJVST should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

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Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015 Apr22;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.

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" 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."

Conflict of interest: All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of poten-

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Abbreviations: Define abbreviations that are not standard in this field in a list to be placed on the tittle page. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the tittle page. Ensure consistency of abbreviations throughout the article.

Main Text

Introduction: Introduction should be as concise as possible, and clearly explain the main objective and hypothesis of the investigation.

Results: Results indicate the results of an original research in a clear and logical sequence. Do not repeat data that are already covered in tables and illustrations. In manuscripts describing more than one animal, all animals should be assigned a case number.

Discussion: Discussion should include the answer to the question proposed in the introduction and emphasize the new and important aspects of the study and the conclusions that follow from them. It could include the implication, application, or speculation of the findings and their limitations, relate the observations to other relevant studies, and links the conclusions with the goals of the study. Recommendations, when appropriate, may be included.

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 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 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 and Results 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.

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

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers conce ntration (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

Data analysis: details for the quantitative or relative analysis.

Use of antibodies: Authors must show that the antibodies are validated and their specificity sis confirmed.

References: Must be up-to-dated and limited to those that are necessary. Lists of references should be given in numerical order in the text, and in the reference list. Please use Vancouver style. To download the Vancouver Style follow the link in the IJVST website which could be used in the Endnote software.

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 diag-

nosed 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.
3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.
4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.
5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;62(1):112-6. Doi: 10.1001/archneur.62.1.112 .
6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015;44(6):387-92.Doi: 10.1001/archneur.62.1.112 .

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).

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Publication Ethics

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manner.

- If the infraction is less severe, the Editor, upon the advice of the Committee on Publication Ethics, sends the author a letter of reprimand and reminds the JAM publication policies; if the manuscript has been published, the Editor may request the author to publish an apology in the journal to correct the record.

- If the author's explanation is unacceptable and it seems that serious unethical conduct has taken place, the matter is referred to the Publication Committee via Editorial board. After deliberation, the Committee will decide whether the case is sufficiently serious to warrant a ban on future submissions.

Post-Publication Discussions and Corrections

This journal allows debate post publication on journal's site, through "Send comment about this article" section to the editor up to one month before final publication. Our mechanisms for correcting, revising or retracting articles after publication depends on the content of the received comment and if the sent comments are useful and applicable for readers/authors, they will be showed under reference section of the articles pages.

Complaint Policy

If the authors disagree with the editorial decision on their manuscripts, they have a right to appeal. Authors who wish to appeal an editorial decision should contact the Editor-in-Chief of the Iranian Journal of Veterinary Science and Technology. In such cases the Editor-in-Chief will review the manuscript, the editorial and peer reviewers' comments and gives his/her decision for accepting or rejecting a manuscript. Editor-in-Chief may, if so required, send the manuscript to a new handling editor for a fresh editorial review and to new reviewer for further peer reviewing. In such case, the final decision maker will be the Editorial board of the journal.

How to Make a Complaint

The procedure to make a complaint is quite simple. The complaint can be made by writing an e-mail to: ijvst@um.ac.ir. All complaints will be acknowledged within a week.

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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