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Kafkas Univ Vet Fak Derg
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Kafkas Univ Vet Fak Derg
Volume: 31, Issue: 6, Year: 2025

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Journal Home-Page: http://vetdergikafkas.org
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Kafkas Univ Vet Fak Derg
Volume: 31, Issue: 6, Year: 2025

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 31, Issue: 6, Year: 2025

CONTENTS

ARTICLE IN PRESS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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REVIEW ARTICLE

Research Progress on the Functions of Non-Structural Protein 2 (NS2) of Classical Swine Fever Virus: A Review

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Abstract

Classical Swine Fever Virus (CSFV) is a significant pathogen that causes swine fever, and its non-structural protein, NS2, plays a crucial role in the viral life cycle. The NS2 protein not only participates in the processing of the viral polyprotein precursor but also significantly affects viral replication, assembly, and infection. In recent years, research on the functions of the NS2 protein has deepened, revealing its self-cleaving enzyme activity, interaction with the NS3 protein, and potential role in viral genome packaging. At the same time, the genetic variation of the NS2 protein and its adaptive changes under selective pressure provide an important theoretical basis for understanding the virus's adaptation mechanisms and vaccine development. This article aims to systematically review the latest research progress on the structural characteristics, processing mechanisms, and functional roles of the NS2 protein of CSFV, in order to provide reference and guidance for the development of future antiviral strategies.

Keywords: Classical Swine Fever Virus (CSFV), Genome packaging, Non-structural protein 2 (NS2), Proteolytic cleavage. Research progress, Viral replication

Introduction

Classical Swine Fever Virus (CSFV), as an important member of the *Pestivirus* genus in the *Flaviviridae* family, poses a serious threat to the global pig industry. According to the ICTV 2017 classification [1,2] (ICTV proposal 2017.010S), the species name of Classical Swine Fever Virus is *Pestivirus C*, whereas according to the ICTV 2022 classification [3,4] (ICTV proposal 2022.007S), it is *Pestivirus suis*. The classical swine fever (CSF) caused by this virus (CSFV) is an important infectious disease in the pig farming industry, characterized by high mortality and high infectivity. It is classified as a Category A notifiable animal disease by the World Organization for Animal Health (The current abbreviation is "WOAH", while the former abbreviation was "OIE") [5,6]. Since its discovery in Ohio, USA, in 1833, this disease has spread widely around the world, causing significant economic losses to the global pig industry [7-9].

In recent years, with the continuous deepening of research on CSFV, scientists have gradually recognized the importance of the NS2 protein in the viral life cycle. The NS2 protein is a hydrophobic membrane-spanning protein with intrinsic self-protease activity. It significantly contributes to the liberation of the NS3 protein [10].

The functions of the NS2 protein are diverse and complex, involving multiple aspects, such as viral replication, assembly, genome packaging, and viral particle formation [11,12]. Studies have shown that the NS2 protein is not only a core component of viral polyprotein processing but also

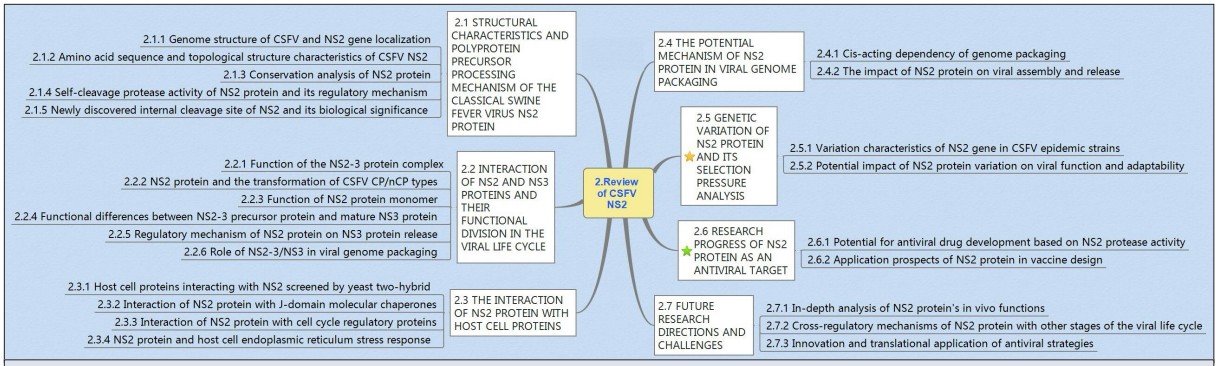

Fig 1. Study scheme on the research review of the NS2 protein of CSFV

influences the maturation and release of NS3 by regulating the cleavage process of NS2-3, thereby promoting effective viral replication [13]. In the early stages of CSFV infection, the NS2 protein fosters the release of NS3 through its self-protease activity, while the mature NS3 is a multifunctional enzyme essential for viral replication, possessing helicase, nucleoside triphosphatase (NTPase), and protease activities. This dynamic protein processing plays a key role at different stages of the viral life cycle, ensuring effective transmission and proliferation of the virus [10].

In recent years, significant progress has been made in the structural and functional studies of the NS2 protein. Through in-depth analysis of the NS2 protein and related molecules, scientists have revealed its multiple roles in the life cycle of CSFV [14,15]. For example, researchers have found that the interaction of the NS2 protein with the cellular protein DNAJC14 is a key regulatory factor for its self-protease activity, and the absence of DNAJC14 may lead to changes in the viral replication mechanism [13]. This finding provides a new perspective for understanding the functions of the NS2 protein, suggesting that there may be different cellular factors involved in regulating the activity of the NS2 protein.

A deeper understanding of the multifaceted functions of the NS2 protein not only aids in uncovering the intricate pathogenic mechanisms underlying CSFV but may also offer significant insights and valuable clues that could be instrumental in the development of innovative antiviral drugs and effective vaccine strategies aimed at combating this viral infection *(Fig. 1)* [16]. With the continuous exploration of the NS2 protein and its role in the viral life cycle, specific inhibitors targeting its functions may be discovered in the future, providing new therapeutic ideas for controlling the spread and infection of CSFV. In summary, the NS2 protein has significant scientific value in the biological research of CSFV, and its in-depth study will help promote the continuous improvement of control strategies for CSFV.

Structural Characteristics and Polyprotein Precursor Processing Mechanism of The CSFV NS2 Protein

Genome Structure of CSFV and NS2 Gene Localization

CSFV belongs to the *Flaviviridae* family and the *Pestivirus* genus, and it exhibits serological cross-reactivity with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) of the same genus [17-19]. The genome of CSFV is a single-stranded positive-sense RNA, approximately 12.3 kb in length, containing a large open reading frame (ORF) that encodes a polyprotein of approximately 3,898 amino acids. This polyprotein is cleaved into four structural proteins (C, E^{rns}, E1, and E2) and eight non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) under the action of host cell and virus-encoded proteases [17].

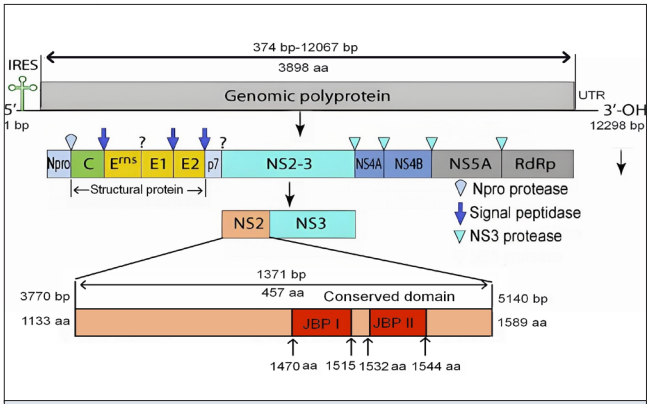

Fig 2. The genome map and NS2 gene location of CSFV. The overall structure of the CSFV genome can be categorized into three distinct components: the 5'-untranslated region (5'-UTR), which encompasses the internal ribosome entry site (IRES); the ORF responsible for polyprotein synthesis; and the 3'-untranslated region (3'-UTR). The NS2 gene is located in the middle of the polyprotein ORF, adjacent to the p7 gene on the left and the NS3 gene on the right. The gene contains two conserved domains, JBP I and JBP II, that bind to Jiv90 (host cellular molecular chaperone)

Among them, the non-structural protein NS2 is a hydrophobic transmembrane protein. Bioinformatics analysis indicates that the full length of the CSFV NS2 gene is 1371 bp, encoding a total of 457 amino acids, with a molecular weight of approximately 52 kDa [20]. To study the function of the CSFV NS2 protein, we mapped the genome structure of the CSFV Shimen virulent strain and the localization of the NS2 gene *(Fig. 2)* [12,15,21]. The NS2 gene resides centrally within the polyprotein ORF, flanked to the left by the p7 gene and to the right by the NS3 gene. This gene encompasses two conserved domains, JBP I and JBP II, which interact with Jiv90, a molecular chaperone of the host cell.

Amino Acid Sequence and Topological Structure Characteristics of CSFV NS2

The amino acid sequence of the CSFV NS2 protein shows high conservation among different strains. Comparative analysis of the amino acid sequences of NS2 proteins from multiple CSFV strains reveals that the conserved amino acid residues are mainly concentrated at the N-terminus and C-terminus, while the amino acid sequence in the middle region is relatively variable [21]. The N-terminus of the NS2 protein contains multiple conserved cysteine residues, which may participate in the formation of intramolecular or intermolecular disulfide bonds, playing an important role in maintaining the structure and function of the protein [22].

Bioinformatics analysis predicts that the NS2 protein has a complex topological structure, containing multiple transmembrane regions. Studies show that the N-terminus of the NS2 protein contains four transmembrane helices (TM1-TM4), and the C-terminus contains one transmembrane helix (TM5). Among them, TM1 - TM4 form a tight hydrophobic domain that anchors the NS2 protein to the endoplasmic reticulum membrane, while TM5 may participate in the interaction of the NS2 protein with other viral proteins or host cell proteins [21].

Conservation Analysis of NS2 Protein

The NS2 protein exhibits a high degree of conservation in CSFV and other *Pestivirus* species, and its structural characteristics play an important role in the viral life cycle. Studies indicate that the NS2 protein contains a key protease active site, a feature commonly found in different *Pestivirus* species [23]. This conservation suggests a core function of the NS2 protein in the viral replication process, particularly in the processing of polyprotein precursors. Specifically, the self-protease activity of the NS2 protein enables it to mediate the cleavage of the NS2-3 precursor, thereby regulating the release of the NS3 protein, which plays an essential role in viral replication and assembly [10]. The conservation of the NS2 protein provides an important basis for understanding its function in the viral life cycle.

Self-Cleavage Protease Activity of NS2 Protein and Its Regulatory Mechanism

The self-cleavage protease activity of the NS2 protein is not only an essential feature of its function but also has a profound impact on the viral life cycle. Through self-protease activity, the NS2 protein can cleave the NS2-3 precursor, releasing the functionally diverse NS3 protein [24]. The NS3 protein is involved not only in viral replication but also plays a vital role in viral assembly and release. Notably, the cleavage activity of the NS2 protein is influenced by different stages of the viral life cycle and cytokines. For example, the action of certain cytokines may enhance or inhibit the cleavage activity of NS2, thereby regulating the efficiency of viral replication and assembly [10]. This regulatory mechanism provides new insights into understanding the biological characteristics of CSFV and offers potential targets for developing antiviral strategies against CSFV.

Newly Discovered Internal Cleavage Site of NS2 and Its Biological Significance

Recent studies have identified a new internal cleavage site of the NS2 protein, mediated by the NS3/4A protease, specifically located at L188-G189. This cleavage site shows a certain degree of conservation among multiple *Pestivirus* species, indicating its importance in viral function [25]. Although the impact of this cleavage site on viral replication and viral particle formation is limited in *in vitro* cell experiments, its function *in vivo* requires further investigation. This finding offers new insights into the multifunctionality of the NS2 protein and may reveal the complex regulatory mechanisms of the virus within host cells. Future research is expected to clarify the specific biological significance of this cleavage site and its role in the viral life cycle, providing a new theoretical basis for the prevention and control of CSFV.

Interaction of NS2 and NS3 Proteins and Their Functional Division in the Viral Life Cycle

Function of the NS2-3 Protein Complex

The NS2 protein exists mainly in two forms after CSFV infects host cells: the NS2-3 protein complex and the NS2 monomer. The NS2-3 complex is a key molecule in the replication and viral particle assembly processes of *Pestivirus* life cycle [26].

In BVDV, the NS2-3 protein complex contains 1140 amino acid residues and has a size of 120 kDa. The NS2-NS3 complex of BVDV can be detected in infected cells, while the production of NS2 and NS3 monomers of BVDV is

completed by the proteolytic activity of the BVDV NS2 protease [27]. Studies on BVDV, which belongs to the same genus as CSFV, indicate that the NS2-NS3 complex is essential for the formation of viral particles [26]. Still, only the NS3 monomer is necessary for viral replication [28,29]. NS3 has helicase [30], NTPase [31], and protease activities [24], making it an essential protein for CSFV genome replication.

The NS2-3 complex is necessary for viral particle formation. Inserting a ubiquitin gene or an internal ribosome entry site between NS2 and NS3 of BVDV can disrupt the NS2-3 complex. This type of mutation does not affect viral replication but leads to defects in the generation of progeny viral particles. However, supplementing the NS2-3 complex with exogenous expression plasmids can restore progeny virus production [26]. Research on CSFV shows that during viral replication, the NS3 protein is released from the NS2-3 complex and joins the replication complex to function as an RNA helicase, thereby participating in viral replication [32,33]. During viral assembly, the NS2-3 complex recruits the NS4A protein to participate in the formation of progeny viruses on the endoplasmic reticulum [34].

NS2-3 can be cleaved by NS2 into NS2/NS3 monomers. Meyers *et al*. discovered the cIns sequence in BVDV [35], and Rinck et al.[36] identified it as the Jiv90 protein. Lackner et al.[37] demonstrated that NS2 has cysteine protease activity, Jiv90 regulates viral replication [38], and the binding domains of NS2 and Jiv90 were identified as JBP I and JBP II, respectively [23]. Balint et al.[39] found that the insertion sequence enhances NS2-3 cleavage. Agapov et al.[26] confirmed that the NS2-3 complex is essential for viral assembly. The NS2-host interaction mechanism still needs further research.

NS2 Protein and the Transformation of CSFV CP/nCP Types

BVDV, a member of the *Pestivirus* genus, has two biotypes: cytopathogenic (CP) and non-cytopathogenic (nCP). The CSFV wild-type strain usually does not produce cytopathic effect (CPE). Genomic recombination can lead to the transformation from nCP to CP [40,41]. commonly seen in the insertion of a ubiquitin gene in the NS2-3 region [42]. In 1998, Meyers G et al.[35] discovered that the CP-type BVDV NADL strain had a 270bp cellular sequence cIns inserted between NS2-3, and its deletion resulted in the loss of CPE. Additionally, Kummerer et al.[43] found that CP type originated from mutations in nCP type, with changes in the NS2 gene being a key pathway. In 1999, Moser et al.[44] constructed a CSFV deletion clone and found that NS2 is non-essential but regulates viral replication. The deletion of the NS2 gene does not affect the replication of infectious RNA in cells; however, the NS2-deleted strain can cause CPE. In 2001 Aoki et al.[45] and in 2004 Aoki et al.[46] found that nCP-type CSFV can assist in the replication of defective particles, and CPE is associated with the accumulation of NS3. In 2007, Moulin et al.[34] confirmed that NS2-3 is essential for viral assembly, and CSFV lacking NS2 cannot complete the packaging of viral particles. In 2008, Gallei et al.[47] constructed a chimeric virus, demonstrating that the chimeric virus's CP-type virulence was weakened. Studies indicate that NS3 promotes the development of CPE, while NS2 may inhibit this effect. In 2011, Lamp et al.[20] found that nCP CSFV maintains low levels of mature NSP by delaying the processing of NS2-3 and downstream NSP, which may be related to its persistent infection characteristics. In contrast, CP CSFV accumulates NSP and causes CPE due to the high expression and rapid processing of NS3.

Function of NS2 Protein Monomer

The NS2 protein is highly conserved, a hydrophobic transmembrane protein located on the endoplasmic reticulum membrane, containing one self-protease responsible for the cis-cleavage at the NS2-3 junction, and its N-terminus can regulate viral replication [12]. Studies show that the different forms of NS2 and NS3 present *in vivo* are closely related to the CPE of the virus [29,39].

Current research on the NS2 protein of CSFV indicates that NS2, when expressed in cells, does not induce apoptosis but can regulate the host cell cycle to arrest in the S phase and upregulate the expression of IL-8 [14,15].

In 2011, Guo et al.[21]'s research showed that the CSFV NS2 protein contains two intrinsic signal peptide sequences, which are involved in the translocation of the NS2 protein to the endoplasmic reticulum. The NS2 protein may also have at least four transmembrane domains. The NS2 protein contains 457 amino acid residues. It exhibits self-cleaving protease activity, which is released from the NS2-3 complex through a cis-cleavage process, and anchors itself to the endoplasmic reticulum via its hydrophobic structure. The amino acid residues His1447 and Cys1512 are essential for maintaining NS2 protease activity [21,24]. NS2 regulates the number of NS3 monomers in the cell through its self-cleavage process, thus preventing excessive RNA replication that leads to the accumulation of viral RNA, causing CPE. Therefore, NS2 acts as a switch for *Pestivirus* replication and assembly [34]. *In vitro* experiments also demonstrate that although NS2 is not related to genome replication, it can extend the half-life of RNA replication complexes. Recent studies on NS2 monomers also show that it can participate in various life processes within the cell. First, NS2 can accelerate the degradation of Cyclin A by the proteasome while increasing the translation level of Cyclin A, leading to the host cell division process being stalled in the S phase, providing an optimized

intracellular environment for viral replication [14]. Second, NS2 can induce endoplasmic reticulum stress responses and activate NF-κB, leading to the upregulation of IL-8 expression, synergistically antagonizing type I interferon responses. Meanwhile, research by Tang et al.[15] proves that CSFV NS2 protein expression can also increase the expression of the anti-apoptotic protein Bcl-2, resisting the apoptosis response induced by MG132, thus playing a role in the inflammatory response and the persistent infection of CSFV.

Functional Differences between NS2-3 Precursor Protein and Mature NS3 Protein

In the life cycle of CSFV, the functions of the non-structural protein NS2-3 precursor and mature NS3 protein exhibit significant differences. The NS2-3 precursor plays a key role in the later stages of viral particle generation, while the mature NS3 protein is primarily responsible for the viral replication process [34]. Specifically, the NS2-3 precursor serves not only as the basis for viral assembly but also as an important bridge in the formation of viral particles, promoting the aggregation and assembly of other structural proteins. Research shows that during the viral life cycle of CSFV, only the uncleaved NS2-3 precursor can effectively support virus production, indicating that the self-protease activity of NS2 plays a crucial role in the viral life cycle [10].

On the other hand, the mature NS3 protein exhibits various enzymatic activities, including helicase [30], NTPase [31], and protease [24]. These functions are essential for viral RNA replication. Studies indicate that NS3 can promote the synthesis and replication of viral RNA through its helicase and NTPase activities. During the replication process, NS3 interacts with other non-structural proteins, such as NS4A, to form an efficient replication complex. This interaction is crucial for ensuring the effective replication of viral RNA and subsequent viral particle formation [48].

In summary, the NS2-3 precursor and mature NS3 protein play different roles in the viral life cycle, with the former focusing on viral assembly and the latter concentrating on RNA replication. This functional division provides an essential basis for understanding the replication and assembly mechanisms of CSFV.

Regulatory Mechanism of NS2 Protein on NS3 Protein Release

The NS2 protein plays a crucial role in regulating the release of the NS3 protein, with its self-protease activity and interaction with cytokines ensuring the timing and efficiency of NS3 release. Specifically, NS2 catalyzes the cleavage of the NS2-3 precursor through its self-protease activity, releasing the mature NS3 protein [10]. The timing of this process is crucial for viral replication and assembly. Studies indicate that the self-protease activity of NS2 is co-regulated by cytokines, and this regulatory mechanism plays a vital role in ensuring the temporal coordination of viral replication and assembly processes [49].

Additionally, the NS2 protein enhances its regulatory ability on NS3 release through interactions with the cell membrane and binding with other non-structural proteins. For example, the interaction between NS2 and NS3 is achieved by forming a membrane-bound complex, and the stability of this complex directly affects the efficiency and timing of NS3 release [50]. Research shows that only by releasing NS3 at the appropriate time can the virus effectively carry out RNA replication and particle assembly. This regulatory mechanism not only improves the efficiency of the viral life cycle but also provides potential targets for future antiviral therapies [50].

In-depth research on the regulatory mechanism of NS2 on NS3 release can provide new insights into understanding the viral life cycle and assist in developing therapeutic strategies against CSFV.

Role of NS2-3/NS3 in Viral Genome Packaging

In the process of genome packaging of CSFV, the NS2-3 precursor and mature NS3 protein play different but complementary roles. Studies show that the mature NS3 protein must be encoded by the same RNA molecule (i.e., cis-acting) to ensure efficient genome packaging. The expression of NS3 is directly related to the efficiency of viral genome packaging; effective genome packaging can only be achieved when NS3 coexists with its encoding RNA [10].

Moreover, the roles of NS2-3 precursor and mature NS3 in genome packaging also show significant differences. The NS2-3 precursor is not only a necessary component required for viral particle assembly, but also provides support for the release of NS3. The mature NS3 primarily handles RNA binding during the packaging process, ensuring the integrity and specificity of the viral genome. In this process, the presence and activity of NS2 assist the function of NS3, ensuring the efficient packaging of the viral genome and the successful assembly of viral particles [13,48].

It is worth noting that in 2019, Dubrau et al.[51] demonstrated that the genus *Pestivirus* can achieve NS2-3 independent virus particle formation through a few key mutations (such as NS2 V439D/T444V, NS3 M132A, NS4A A48T, and NS5B D280G). These mutations coordinate the transition between RNA replication and virus packaging by regulating the conformation of the NS3/4A complex and the function of NS5B.

Based on these findings, the NS2-3 precursor and mature NS3 protein, along with their conserved amino acid

residues, play an indispensable role in the packaging of the virus genome. Their collaboration ensures the assembly specificity and integrity of the virus particles, providing important scientific evidence for understanding the biological characteristics of the CSFV and developing corresponding antiviral strategies.

The Interaction of NS2 Protein with Host Cell Proteins

Host Cell Proteins Interacting with NS2 Screened by Yeast Two-Hybrid

Kang et al.[52] screened multiple host cell proteins interacting with NS2 using the yeast two-hybrid system: GOPC, HNRNPH1, DNAJA1, ATP6, CSDE1, CNDP2, FAN CL, TMED4, DNAJA4, MOAP1, and PNMA1. These proteins are primarily associated with apoptosis, stress response, redox balance, and metabolism.

Interaction of NS2 Protein with J-Domain Molecular Chaperones

In CSFV, the self-cleavage activity of the NS2 protein depends on the assistance of the host protein Jiv (also known as a J-Domain protein interacting with virus protein). The Jiv90 fragment serves as the active form of Jiv and plays a key role in viral replication. Studies have found that Jiv90 is directly associated with the function of CSFV NS2: overexpression of Jiv90 significantly promotes viral replication, while knockdown of its expression inhibits viral replication. This interaction occurs in the cytoplasm (not the endoplasmic reticulum) and affects the efficiency of viral RNA replication [11].

Interaction of NS2 Protein with Cell Cycle Regulatory Proteins

The normal operation of the cell cycle is crucial for cell growth, proliferation, and differentiation. Viral infection often interferes with the cell cycle process to meet its own replication and proliferation needs. After cells are infected with CSFV, the expression of the NS2 protein is upregulated, which inhibits the activity of CDK1 by interacting with cell cycle regulatory proteins, leading to the cell cycle being unable to progress normally and stagnating in the S phase [14]. The S phase of the cell cycle is the period of DNA synthesis, and the virus may utilize the abundant DNA synthesis materials and related enzymes in the host cell during this period to replicate its own genomic RNA. Meanwhile, cells stalled in the S phase show reduced sensitivity to interferons (Interferon, IFN), which may help the virus evade the host's immune clearance and promote viral infection and proliferation [53].

NS2 Protein and Host Cell Endoplasmic Reticulum Stress Response

In 2010, Tang et al.[54] constructed subclones of different segments of NS2 and transfected host cells. Through confocal microscopy, they found that the NS2 protein is localized in the endoplasmic reticulum of host cells and contains at least two internal signal peptide sequences and four transmembrane regions. The transfected NS2 protein is rapidly degraded in host cells; however, the proteasome inhibitor MG132 can prevent the rapid degradation of the NS2 protein. Their research also found that the CSFV NS2 protein can induce an endoplasmic reticulum stress response in host cells and activate the nuclear transcription factor NF-κB, suggesting that the rapid degradation of CSFV NS2 protein by the proteasome may be related to the endoplasmic reticulum stress response.

The Potential Mechanism of NS2 Protein in Viral Genome Packaging

Cis-Acting Dependency of Genome Packaging

Genome packaging is a crucial step in the viral life cycle, involving the effective encapsulation of the viral genome. For the CSFV, the NS2 protein plays a key role in this process. Studies have shown that genome packaging depends on the cis-encoded mature NS3 protein, which can prevent the encapsulation of defective genomes, thereby improving the quality of viral particles. This was validated through trans-packaging experiments of viral subgenomes, which showed that genome packaging could only proceed effectively when the NS3 protein is encoded by the RNA molecule itself [10]. Although the NS2-3 precursor can provide support in the trans mode to facilitate the assembly of viral structural proteins, the encapsulation of the genome requires the cis expression of NS3. This finding highlights the pivotal role of NS3 in viral genome packaging and suggests a more intricate NS2-3/NS3 functional model, underscoring the complexity of the viral packaging mechanism. In this process, NS3 is not only a multifunctional enzyme with helicase and protease activities but also participates in genome encapsulation through its mature form. The self-cleavage process of NS2 is also considered a crucial mechanism regulating the release of the NS3, thereby ensuring the efficient release of mature NS3 during the early stages of the viral life cycle. This regulatory mechanism may serve as a downstream quality control to prevent the packaging of defective genomes and coordinate the encapsulation of RNA molecules before membrane acquisition [48,49]. Therefore, the role of NS2 in genome packaging is not limited to providing precursors but also indirectly determines the quality and efficacy of viral particles by influencing the maturation and release of NS3.

The Impact of NS2 Protein on Viral Assembly and Release

The NS2 protein also plays an important regulatory role in the processes of viral assembly and release. Research has shown that the NS2 protein indirectly affects the assembly and release of viral particles by regulating the release of the NS3 protein. Specifically, the proteolytic activity of NS2 and its interactions with other proteins may be involved in the formation of the viral membrane and the maturation of viral particles. In studies of HCV, NS2 is not only responsible for the self-cleavage of the NS2-NS3 precursor but also promotes the recruitment of envelope protein E2, thereby affecting viral assembly [55]. Furthermore, interactions between NS2 and other non-structural proteins have also been shown to be crucial in viral assembly. For example, the direct interaction between NS2 and NS3 proteins, as well as the binding with envelope proteins such as E1 and E2, plays an important role in connecting and stabilizing structures during the formation of viral particles. Through these interactions, NS2 helps establish a complex protein network, providing the necessary support for effective viral assembly [56,57].

Overall, the role of the NS2 protein in viral assembly and release is multifaceted. It not only affects packaging efficiency by regulating the release of NS3 but may also promote the maturation and release of viral particles through interactions with other key proteins. Understanding the comprehensive function of NS2 in the viral life cycle will provide an important theoretical basis for developing new antiviral strategies.

Genetic Variation of NS2 Protein and Its Selection Pressure Analysis

Variation Characteristics of the NS2 Gene in CSFV Epidemic Strains

Genomic analysis of Japanese CSFV epidemic strains from 2018 to 2020 revealed significant non-synonymous mutations in the NS2 protein, indicating that this gene is subject to positive selection. This phenomenon reflects the genetic variation that occurs in CSFV during the epidemic process, allowing it to adapt to the host environment and strengthen its transmission capability. Compared to highly conserved non-structural proteins such as NS3 and NS4A, the variation characteristics of the NS2 protein are more pronounced, which may be closely related to its role in viral adaptive adjustment. Specifically, as a multifunctional non-structural protein, NS2 is involved in viral replication, assembly, and immune escape, and its variation may provide the virus with new adaptive advantages. In the variations of the epidemic strains, studies found that most mutation sites related to NS2 are located within its functional domain. These mutations not only affect the structure and function of NS2 but may also influence its interactions with other non-structural proteins, thereby affecting the overall biological characteristics of the virus [22].

Moreover, the variation characteristics of the NS2 gene in epidemic strains also indicate that the virus may adapt to new ecological niches through selective mutations in different host environments. The accumulation of these mutations may support the transmission and pathogenicity enhancement of CSFV [58,59]. The emergence of positive selection, especially during periods of high epidemiological incidence, further highlights the importance of NS2 in the CSFV life cycle and its key role in adaptive evolution.

Potential Impact of NS2 Protein Variation on Viral Function and Adaptability

The variation of the NS2 protein not only affects its own function but may also have profound effects on the overall adaptability of the virus. Firstly, these variations may alter the protease activity and self-cleavage efficiency of NS2, changing its interactions with other non-structural proteins such as NS3. For example, the NS2 protein is believed to play an important role in viral assembly, and its self-cleavage function directly affects the release of NS3, which is an indispensable component in the viral replication process. Therefore, variations in NS2 may indirectly affect the virus's replication capability and assembly efficiency by influencing its self-cleavage process [55].

Secondly, variations in NS2 may also facilitate the virus's escape from immune surveillance, thereby enhancing its adaptability in diverse host environments. This adaptive change may lead to the virus exhibiting different pathogenicity and transmission capabilities in different hosts. For instance, specific mutations may enable the virus to replicate more easily in specific hosts or enhance its resistance to the host immune system, thereby increasing the virus's transmission potential and pathogenicity [60]. In summary, the genetic variation of the NS2 protein is not only a result of CSFV adaptive evolution but also a key factor for its successful transmission in different hosts. Future research should further investigate the specific effects of these variations on viral function, providing new insights for the development of vaccines and therapeutic strategies against CSFV.

Research Progress of NS2 Protein as An Antiviral Target

Potential for Antiviral Drug Development Based on NS2 Protease Activity

The NS2 protein of CSFV plays a vital role in the virus's life cycle, particularly in the cleavage and maturation of viral proteins. Studies have shown that the NS2 protein possesses self-protease activity, which facilitates the cleavage between NS2 and NS3, a process crucial for viral replication [10]. Therefore, the self-protease activity of NS2 provides a highly promising target for designing specific inhibitors. By inhibiting NS2-3 cleavage, it is possible to effectively block viral replication, thus offering new ideas for antiviral drug development.

In current research, the screening and structural optimization of small-molecule inhibitors and protease inhibitors have become hot topics. Many researchers are exploring compounds that can target the NS2 protein, which not only need to have high affinity for NS2 but also effectively inhibit its enzymatic activity, thereby reducing the virus's infectivity. For example, by utilizing high-throughput screening technology, researchers have identified several small-molecule inhibitors that can significantly reduce the replication levels of CSFV and Hepatitis C Virus [61]. Additionally, with the advancement of structural biology, researchers have begun to utilize computer simulation and molecular docking techniques to design more specific and efficient NS2 inhibitors, which are expected to play a crucial role in future clinical applications.

In summary, the development of antiviral drugs based on NS2 protease activity has significant theoretical significance and practical application potential. With a deeper understanding of NS2's functions, it is expected that more effective antiviral drugs against CSFV will be developed in the future, providing new solutions for controlling CSFV outbreaks [62].

Application Prospects of NS2 Protein in Vaccine Design

In 2002, Armengol [63] discovered that NS2 contains T cell epitopes. The conservative nature and key functions of the NS2 protein make it an important candidate target for vaccine design. During the vaccine development process, the NS2 protein, as a non-structural protein, plays a crucial role in the virus's life cycle, especially during the replication and assembly stages. Research has found that the NS2 protein is highly conserved in CSFV, indicating that it has a relatively stable structure and function across different viral strains, providing a solid foundation for its application in vaccine design [21].

Research on NS2 mutation sites helps improve the broad-spectrum and immunoprotective effects of vaccines. By analyzing the variations of NS2 in different viral strains, researchers can identify key immunogenic epitopes that can induce a broad immune response in the host. For instance, using modern bioinformatics tools, researchers can design recombinant vaccines containing multiple conserved epitopes, which can not only enhance protection against specific viral strains but also improve cross-protection against other related viruses [64].

Moreover, studies have shown that attenuated CSFV strains containing specific NS2 modifications (leading to high expression of NS3) can induce high levels of specific neutralizing antibodies (humoral immunity) in natural host pigs, demonstrating their potential for vaccine development [47]. Future research can focus on optimizing the development of CSFV attenuated strains of NS2 protein to enhance the immunogenicity and safety of vaccines, thereby providing a more solid foundation for CSFV vaccine development [65].

In conclusion, the application prospects of the NS2 protein in vaccine design are broad. With advancements in science and technology, vaccines targeting the NS2 protein are expected to provide new strategies and tools for controlling CSFV outbreaks in the future.

Future Research Directions and Challenges

In-Depth Analysis of NS2 Protein's *In Vivo* Functions

The NS2 protein plays an important role in the life cycle of CSFV, and an in-depth analysis of its *in vivo* functions is a key focus of future research [66]. To reveal the biological significance of NS2 intramolecular cleavage and its regulatory mechanisms, researchers can utilize animal models and *in vivo* infection experiments to explore NS2's functions. Specifically, researchers can use transgenic mice or transgenic pig models to effectively observe the impact of NS2 on host immune responses and NS2's role in viral replication. Additionally, in-depth studies on the interactions between NS2 and host cell factors can reveal how these NS2-host cell factor interactions affect the virus's pathogenicity. Therefore, exploring these interactions and their mechanisms is important. This will help us understand the specific role of NS2 in viral pathogenesis.

Cross-Regulatory Mechanisms of NS2 Protein with Other Stages of the Viral Life Cycle

The NS2 protein plays a role in viral replication. It may also participate in cross-regulatory mechanisms during other

stages of the viral life cycle, including assembly and release. Researchers should focus on the regulatory network of NS2 during these stages to gain a deeper understanding of its functions. For example, high-throughput omics technologies can identify key host proteins that interact with NS2 and analyze how these interactions affect different stages of the viral life cycle. Furthermore, structural biology techniques can also elucidate the multifunctional mechanisms of NS2, revealing its specific roles in viral assembly and release. Existing studies have shown that specific amino acid residues in the Influenza A Virus NS2 protein significantly impact the virus's survival and replication. This indicates that NS2's role in the viral life cycle extends beyond a single stage [67]. Therefore, a thorough understanding of NS2's regulatory roles at various stages of the viral life cycle will provide crucial insights for the development of new antiviral strategies.

Innovation and Translational Application of Antiviral Strategies

Developing new antiviral drugs and vaccines based on the functional characteristics of the NS2 protein is crucial for effective treatment [62]. This approach represents an important direction for future research. Researchers can utilize the unique structure and functions of the NS2 protein to design specific inhibitors targeting the NS2 protein, effectively blocking viral replication and transmission. Additionally, applying NS2-related research findings in clinical and pig farming settings is crucial for controlling the spread of CSFV. For instance, interfering with the interaction between NS2 and host cell factors may effectively reduce the virus's pathogenicity. This approach offers new insights for developing innovative antiviral strategies. At the same time, integrating modern biotechnologies, such as gene editing and vaccine vector technologies, can enhance the immunogenicity and safety of vaccines. This improvement will promote their application in the farming industry. Through these innovative studies, the threat of CSFV to the swine industry is expected to be significantly reduced in the future. This reduction will contribute to animal health and food safety.

Conclusion

CSFV, as an important viral animal disease agent, affects not only the pig farming industry but also involves food safety and public health. In recent years, research on the non-structural protein NS2 of CSFV has gained increasing attention, and the revelation of its multiple key functions in the viral life cycle marks a significant milestone in our understanding of this virus. The core role of NS2 in polyprotein processing, NS3 protein release, and viral genome packaging highlights its importance in viral biology.

Recent studies have shown that the discovery of new cleavage sites within the NS2 protein further enriches our understanding of its multifunctionality. These cleavage sites not only provide new perspectives for studying the viral life cycle but also offer a biological basis for the virus's adaptive evolution. As observed in various studies, the genetic variations of NS2 reflect the selective pressures the virus encounters in the host environment, providing important evidence for vaccine design and the development of antiviral strategies. By deeply analyzing the mechanisms of NS2's variations, researchers can better predict the virus's mutation trends, thereby enhancing the effectiveness and durability of vaccines.

As research on NS2 as an antiviral target continues to deepen, future studies are expected to promote the development of new antiviral drugs and vaccines. The multifunctionality of NS2 makes it an ideal research subject, capable of providing key clues to reveal the virus's pathogenic mechanisms and transmission pathways. To achieve this goal, further *in vivo* functional validation and mechanism analysis will be the focus of future research. Researchers need to establish more comprehensive model systems to fully reveal the specific roles of NS2 in the pathogenicity and transmission of CSFV, providing theoretical support and practical guidance for controlling CSFV.

In the current research context, balancing different research viewpoints and findings has become an important challenge we face. Although existing studies provide multifaceted evidence for NS2's functions, caution must be exercised in experimental design and data interpretation. Different research groups may employ different experimental methods and models, resulting in discrepancies in understanding the functions of NS2. Therefore, in future research, establishing unified experimental standards and data analysis frameworks will help integrate different research findings and form a more consistent scientific consensus.

In summary, the NS2 protein plays a crucial role as the core of CSFV research. By exploring its multiple functions and roles in the viral life cycle, we can not only broaden our scientific understanding of CSFV but also lay the foundation for developing effective prevention and control strategies. Future research should focus on addressing current scientific issues and continuously advancing prevention and treatment strategies for CSFV.

Highlight Keypoints

1. Subcellular localization and ER Stress induction: The NS2 protein is mainly localized in the host cell endoplasmic reticulum, activating the NF-κB signaling pathway by inducing endoplasmic reticulum stress, leading to the degradation of Cyclin A protein.

2. Cell cycle regulation: The NS2 protein promotes the degradation of Cyclin A through the proteasome pathway, causing cell cycle arrest at the S phase and significantly inhibiting the proliferation activity of host cells.

3. Protease activity and viral replication: NS2 has its own protease activity, capable of cleaving the NS2-3 precursor protein into NS2 and NS3 monomers, a process that is crucial for viral RNA replication.

4. CPE: NS2 regulates viral replication and inhibits the development of CPE; the absence of the NS2 gene does not affect the replication of infectious RNA in cells, but NS2-deficient strains can cause CPE.

5. Virulence-related functions: The cleavage efficiency of the NS2-3 protein is directly related to the pathogenicity of the virus and is one of the key molecules determining the virulence of CSFV.

Declarations

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Review Article

Nipah Virus: An Emerging Zoonotic Threat with Pandemic Potential, Therapeutic Control and Vaccine Development

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Abstract

Throughout history, viral outbreaks of varying intensities and frequencies have caused disaster and terror in the world. The Nipah virus (NiV), which is extremely virulent, has a high case fatality rate, high pandemic potential, and a contagious virus outbreak of zoonotic origin. This encapsulated virus poses a significant risk of frequent outbreaks in Southeast Asia, and its glycoproteins are necessary for it to enter the host cells. Both neurological and respiratory symptoms are associated with pathogenesis. Like monoclonal antibodies and ribavirin antiviral drugs used as potential remedies, but the treatment is modest. Preventive approaches, such as stringent infection control protocols in hospitals and healthcare facilities and population-wide interventions, can help control NiV outbreaks. Prospects for the future suggest that to improve preparedness, there is a need to put a lot of effort into the development of vaccines and preparing antivirals. A comprehensive strategy for addressing NiV should include community education, rigorous surveillance, and epidemiological surveys. A concerted One Health approach that supports human, animal, and environmental surveillance is also essential for NiV management and prevention. In this review, the outbreak of NiV, along with the routes of transmission, prevention and control strategies used, potential causes of the outbreaks, and the precautions that private-public initiatives should take to ensure a lower incidence of disease are discussed.

Keywords: Nipah virus, Hendra virus, Henipavirus, zoonosis, Bat-borne disease, Pathogenesis, Pandemic potential

Introduction

The NiV is a highly contagious virus, belongs to the family *Paramyxoviridae* and genus *Henipavirus*, which has become one of the most significant zoonotic risks to public health over the last 20 years. It is an emerging, pathogenic virus that has been causing outbreaks in South Asia on an annual basis, resulting in lethal respiratory and neurological infections [1]. In 1998, an unknown cause caused several encephalitis outbreaks in Perak, Malaysia. At the start, this outbreak was considered Japanese encephalitis (JE) because the disease showed almost the same signs and symptoms clinically. But after keen research, the disease-causing virus was named NiV in 1999, after the name of the Nipah River, according to the Centers for Disease Control and Prevention (CDC) and the Malaysian Ministry of Health [2]. Fruit bats of the genus *Pteropus* serve as the virus's natural reservoir hosts and are essential to its maintenance and spread. Humans are frequently exposed to contaminated food sources, such as raw date palm sap, through direct contact with diseased animals, or, most concerning, through human-to-human transmission. After being discovered, the NiV was also found in many other countries that were involved in trade with Malaysia. It is highlighted due to its highly zoonotic impact

on people and its immense ability to create a pandemic outbreak [3]. It has been defined among the top 10 emerging contagious viruses. NiV is classified as a Category C priority infection by the World Health Organization (WHO) and is still a potential candidate for the next possible pandemic outbreaks, with a case fatality rate that can vary from 40% to up to 75% depending on the outbreak. Clinically, a NiV infection can present with a wide range of symptoms, from mild fever, illness, or no symptoms to acute respiratory syndrome and lethal encephalitis [4]. Survivors frequently experience neurological sequelae, which increases the impact on public health. Crucially, the absence of efficient antiviral therapy has compelled the use of supportive care exclusively, which has minimal effect on halting the progression of the illness or lowering death. It requires immediate research and development in case of a public health emergency by the WHO, the United Kingdom Vaccine Network, and the Coalition for Epidemic Preparedness Innovations (CEPI), which have identified it as a priority to develop a vaccine against NiV [5]. The pandemic potential, epidemiology, pathophysiology, methods of transmission, zoonotic potential, therapeutic control strategies, and the present state of vaccine research, including its obstacles and advancements, are discussed here.

Etiology

NiV is a negative-sense, ssRNA enveloped, pleomorphic, spherical, and thread-like virus that belongs to the family *Paramyxoviridae* and genus *Henipavirus*. Other members of this family include measles virus, Newcastle disease virus, Mumps, parainfluenza, and Hendra virus (HeV). NiV is also closely related to the Hendra virus. The complete linear genome of NiV is approximately 18.2kb nucleotides [6]. The NiV genome is a non-segmented, negative-sense RNA molecule encoding six major structural proteins, including RNA polymerase or large protein (L), matrix protein (M), fusion glycoprotein (F), attachment glycoprotein (G), phosphoprotein (P), and nucleoprotein (N). It also encodes three non-structural proteins, including C, V, and W, which are produced from the P gene and play roles in viral virulence. So, F and G proteins play a role in the virion-cellular attachment and, afterward, entry into the host cell. The N, P, and L proteins are involved in the viral RNA attachment that results in the virus ribonucleoprotein (vRNP) [7]. Two main, different genotypes of NiV were identified by phylogenetic analysis: NiV-Malaysia (NiV-MY), which is recognized in both Malaysia & Cambodia, and NiV-Bangladesh (NiV-BD), which is recognized in Bangladesh as well as India. Additionally, compared to NiV-BD, NiV-MY genotypes have demonstrated greater virulence, which is directly correlated with the clinical appearance, pathogenicity, mode of transmission, and severity of the disease [8]. The morphology of the NiV is shown in *Fig. 1*.

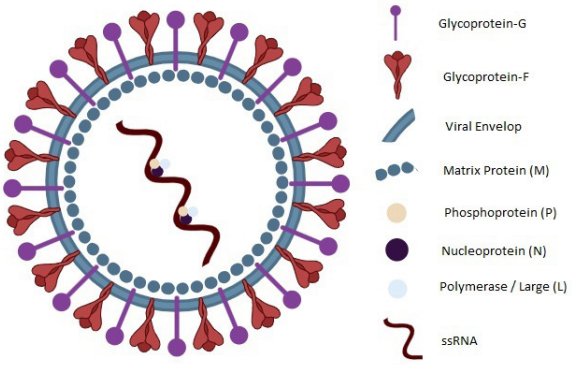

Fig 1. Morphology of NiV. Viral envelope, single-stranded RNA, 6-structural protein: Attachment protein (G), Fusion protein (F), Matrix protein (M), Phosphoprotein (P), Polymerase protein (L), Nucleoprotein (N)

Epidemiology and Pandemic Potential

NiV is a renowned zoonotic viral disease that spreads from animals to people and represents a great threat to both animal and public health. The epidemiology is influenced by the interactions between intermediate animal species, reservoir hosts, human social behaviors, and ecological shifts. NiV also has a high pandemic potential and spreads throughout the South and Southeast Asia [9]. In 1998, the first epidemic outbreak was observed in the pigs in Malaysia. The second outbreak of NiV occurred in a small town in Negri Sembilan in the winter of 1999, and the third and largest outbreak took place near Bukit Pelandok, one of the major pig farming settlements. In this region, the target of NiV infection was pigs, which subsequently spread to humans. They showed the signs of acute respiratory distress and encephalitis [3]. Approximately 265 cases of acute NiV encephalitis were reported, out of which almost 105 people died. So, the infected pigs were exported to other countries by Malaysia, and the NiV disease disseminated there. Beyond Malaysia, NiV cases were reported in neighboring countries, including Singapore, the Philippines, and South Asia (Bangladesh and India), where the cases were reported almost annually [6]. NiV infection reached Singapore in the latter part of February 1999. Since 2001, periodic NiV outbreaks have also been reported in Bangladesh and India, and by 2014, the virus had also infected other countries in South Asia [10]. As Bangladesh is an Islamic country, it didn't import pigs as a source of food. Therefore, the disease was spread to humans through the consumption of contaminated fruits. The virus was believed to have been transmitted by infected horses in the Philippines, where 17 cases with the symptoms were noted. The mortality rate of acute encephalitis was higher at 82 percent. There are also endemic nations such as Thailand, Cambodia, Indonesia, Madagascar, and Ghana. Compared to past infectious

outbreaks, NiV had a higher fatality rate [5]. It is highly virulent and is studied under biosafety level 4 (BSL-4) in a laboratory that is safe for zoonotic emerging infectious diseases. The pandemic potential of NiV is becoming much more significant. It is more likely to spread as a pandemic due to a number of factors, including human sensitivity to the virus and its quick person-to-person transmission. Climate change has also been a concern, with deforestation the risk of occurrence of human-animal contact increases and the chances of spreading of NiV to other tropical regions of the world escalates [11].

Pathogenesis

In humans, NiV enters the body through ingestion or breathing. Epithelial cells, especially those located in the bronchiole, are the first replication sites and may serve as a means of diagnosing NiV early in the course of infection. The bronchi and, in some cases, the alveoli have been found to contain viral antigens. Acute respiratory distress syndrome (ARDS) like illness results from the release of cytokines from the affected respiratory tract epithelium caused by NiV infection [12]. In the later stages, the airway epithelium also secretes inflammatory mediators, including interleukin and granulocyte-colony stimulating the tissues. In both the early and late stages of infection, respiratory or urinary tract epithelial cells play a critical role in viral replication, particularly when it comes to viral shedding and transmission through urine and airway secretions [13].

The virus enters endothelial cells of the surrounding vessels (capillaries) from the respiratory epithelium. As the illness progresses, the virus enters to the bloodstream. Multi-organ failure can result from attacks on the brain, in addition to the pulmonary, digestive, and excretory systems [14]. NiV enters the central nervous system (CNS) through the choroid plexus, a network of blood vessels in the cerebrum. A number of neurological issues can arise as a result of this dissemination of infection across the blood-brain barrier (BBB). Necrosis can result from the presence of virus particles in the central nervous system. Over time, the infection can also propagate to the CNS through the olfactory bulb. Finally, the virus travels down the olfactory tubercle across the entire ventral brain [15]. The pathogenesis of the NiV is shown in *Fig. 2*.

Viral membrane integration with the host cell membrane is made possible by the viral initial attachment to cell surface receptors (ephrin-B2/B3). Transmembrane glycoproteins, which are encoded by all *Paramyxovirus* members, aid in the attachment and fusion of the virus, which in turn allows the virus to enter cells and disseminate during the early phases of infection [16]. Two kinds of envelope glycoproteins are seen on the surfaces of NiV, referred to as G-glycoprotein for attachment and F-glycoprotein for pH-independent fusion activities. The direct transmission of the pathogenic virus from affected to unaffected adjacent cells is mediated by these glycoproteins, which also cause cytopathogenicity. The cytopathic impact of NiV infection causes endothelial cells to develop membrane fusion-mediated syncytia, which is triggered by these two viral glycoproteins. A hallmark that sets NiV illness apart is the development of symptoms in the endothelial cells of the blood vessels [17].

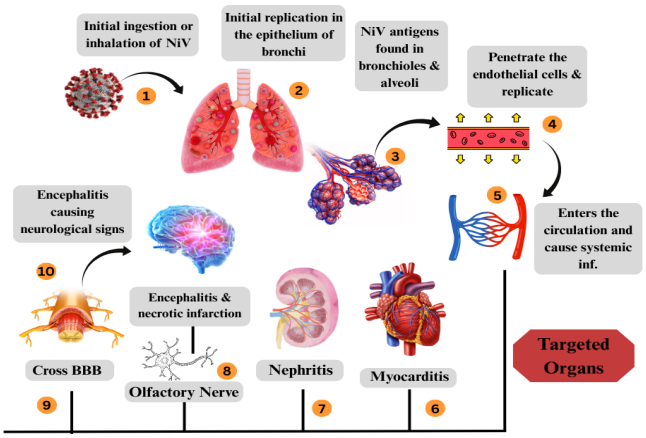

Fig 2. Pathogenesis of NiV. 1. Virus enters the epithelial cells of 2. Bronchioles (Lungs) 3. Viral antigens found in alveoli 4. Penetrate into endothelial cells & replicate 5. Enters circulation 6. Myocardial infarction 7. Renal dysfunction 8. Olfactory nerve leads to encephalitis 9. Crossing the BBB causes neurological signs

Henipaviruses, which belong to the *Paramyxoviridae*, such as NiV, attach to host cell receptors. It's interesting to note that NiV proteins engage alternative host cell receptors because their glycoproteins lack hemagglutination and neuraminidase activity. In contrast, other *Paramyxoviruses*, including *Rubulaviruses, Respiroviruses,* and *Avulaviruses*, bind to receptors that contain sialic acid and exhibit neuraminidase activity. Human neurons, sinus linings of lymphatic nodes, vascular cells, the spleen cells, and placental tissue, arteries surrounding smooth muscle, and airway epithelial cells all express ephrin-B2 receptors; lymphoid cells and the central nervous system express ephrin-B3, a substitute entry receptor [18].

Transmission and its Zoonotic Spillover

Like the related *Hendra virus, Lyssaviruses, Filoviruses,* and *Coronaviruses,* the naturally occurring reservoir host of the NiV is the *Pteropus* fruit bats. Fruit bats (sometimes called megabats) belong to the *Pteropodidae* family. The fruit bats, also known as flying foxes, are primarily responsible for the spread of NiV. Although NiV is a contagious and zoonotic virus, it may potentially spread from person to person through close contact and through either direct or indirect contact with infected animals or their body fluids, like a bat's urine or saliva [19]. The pigs and horses frequently serve as intermediate hosts for NiV. As the outbreak occurred in

Malaysia and its transmission was linked to infected pigs, but in the case of Bangladesh, the pandemic outbreak was due to the consumption of the contaminated date palm sap, and in the Philippines, the infected horses play a role in this zoonotic impact of the NiV disease [20]. The following are the main transmission routes:

People can get the disease by coming into close contact with sick pigs and bats or their waste products, including their urine, feces, or saliva. For a specific period of time, NiV can endure on surfaces in the environment. Transmission can occur if people touch their mouth, nose, or eyes after coming into contact with contaminated surfaces, tools, or items without practicing good hand hygiene, such as people climbing on the date palm tree [21]. In several outbreaks, raw date palm sap tainted with infected fruit bat pee or saliva was consumed by the susceptible host, leading to the spread of the NiV. For a specific period, the virus may survive in date palm sap, making it a possible source of outbreak. The NiV may spread from person to person via direct contact with an infected individual's body fluids, including blood, urine, saliva, and respiratory secretions. The major problem during epidemics is this kind of transmission, especially in medical facilities where exposed patients or their tainted medical equipment may be present [22]. Mistakes in infection control procedures have led to NiV epidemics in medical facilities. When providing treatment for patients infected with the NiV, healthcare professionals and caregivers run the risk of contracting the disease if they fail to take the proper measures, such as wearing personal protective equipment (PPE) [23]. The transmission and zoonotic potential of NiV is shown *Fig. 3*.

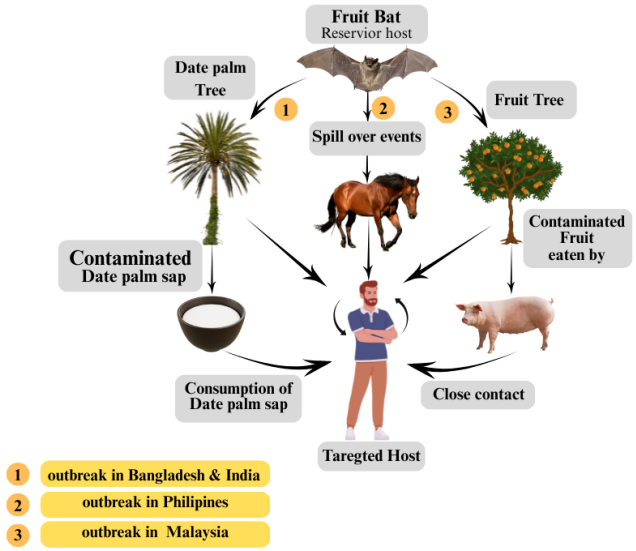

Fig 3. Transmission of NiV and its Zoonotic Spillover. 1. Outbreak in Bangladesh & India by direct contact and consumption of contaminated date palm sap, 2. Outbreak in the Philippines due to the consumption of raw meat of affected horses, 3. Outbreak in Malaysia by contact with infected pigs and fruit bats

Clinical Manifestations

There are several clinical manifestations caused by NiV infection in both humans and animals, depending on the basis of the varying intensities of exposure to the infection.

Humans

Although the NiV is most recognized for its effects on the neurological system, and may also harm the respiratory system. The incubation period for the NiV is 4-45 days. The primary symptoms of NiV are fever, headache, respiratory discomfort, muscle aches, vomiting, poor coordination, disorientation, and uncontrollable walking [24]. There could be bleeding from the gut, renal injury, and the development of septicemia. In extreme cases, the seizures are followed by encephalitis within 24 to 48 hours, which ultimately leads to coma. Approximately 14% to 29% of individuals in Malaysia were reported to have respiratory problems at the time of the initial outbreak [25].

Animals

NiV shows a vast diversity in the viral disease, mostly in pigs or some species of bats. A series of clinical manifestations that cannot be fully understood without the complications of the viral dynamics in the intermediate hosts, as well as in the natural reservoirs.

Various clinical manifestations may occur in pigs, which are intermediate hosts of the NiV infection in humans. These symptoms indicate the characteristic manifestation of the virus, which is to attack the respiratory system, causing difficulty in breathing and frequent coughing [26]. More insights on the virus-to-host interaction can be obtained through neurological manifestations, including apparent trembling, muscle weakness, and poor coordination. It is important to note that miscarriage and stillbirth in pregnant sows are linked to infections caused by NiV in pigs. This is the fact of this virus that it even leads to unexpected and sudden death of the infected pigs without apparent clinical manifestation beforehand [27]. Fruit bats are literally the ones referred to as natural reservoir hosts of the NiV. Strikingly, these bats are unable to show clinical signs. They incubate the virus in their body fluids and waste products, and in doing so, they become the main source of virus transmission. This special attribute highlights the effects of bat-to-animal or bat-to-human through contaminated excretions and secretions [28].

Diagnostic Approaches and Laboratory Confirmations

For the purposes of reversing the high mortality rate associated with NiV infection, the identification of the disease early and accurately is a must. To meet this end, a comprehensive diagnostic procedure involves taking a range of specimens from infected animals and humans.

Samples collected in the case of human patients include cerebrospinal fluid (CSF), urine, blood, throat, and nasal swabs used in diagnosis. In the same breath, diseased animals provide excellent diagnostic specimens that could be used in the isolation and detection of NiV, including their kidney, lungs, and spleen [29]. The NiV diagnostic tests should be performed at highly controlled and dedicated institutions, especially in Biosafety Level 4 (BSL4) laboratories. The RT-PCR tests of the blood, urine, nasal passage, throat, and cerebrospinal fluid can be carried out to detect early development of the NiV infections. Other diagnostic options also include DNA amplification, DNA sequencing, immunofluorescence test, histopathology, viral isolation, neutralization, and high-throughput to carry out complete genome sequencing of NiV infecting individuals and animals [30]. Following WHO and conventional diagnostics of NiV, PCR is the most sensitive procedure to detect an active NiV disease. Nevertheless, NiV-specific IgM ELISA is an alternative serological test in conditions where PCR is inaccessible. ELISA forms a reliable technique in the detection of NiV, though with low sensitivity and specificity compared to molecular detection [31].

Prevention & Control Measures

As the NiV may have various implications, prevention of its infection is essential. As NiV is transmitted to humans through animals, control measures such as avoiding contact with infected animals and secretions of the fruit bats should be taken. Public health measures are necessary in order to slow the spread, including isolating the patients who have been confirmed. Additionally, a quarantine policy might be implemented for those who have had close contact with the reported cases, and symptom observation could be conducted to lessen the chance of transmission [32]. To prevent the transmission of a disease, it is critical to undertake control measures, especially in the case of healthcare workers (HCWs). This has meant that, due to the lessons learnt through previous outbreaks such as the Ebola virus and severe acute respiratory syndrome (SARS), strong recommendations are set regarding the protection of HCWs. An efficient infection prevention and control plan incorporates standard precautions, strict hand hygiene, and personal protective equipment (PPE) use as its guiding principle. These precautions are necessary when handling all patient care operations, even those that make aerosols [33]. This can include separating sick patients in the same rooms or those with single-patient rooms to minimize contact with vulnerable persons. In regions prone to viral epidemics, healthcare facilities ought to be prepared to handle NiV cases. This preparedness also involves broad screening and admissions, effective triage systems. Standard precautions must be observed in every respect of medical care, such as how a patient is handled, collection of the samples, cleaning, and disposal of wastes [34].

Therapeutic Interventions

Currently, there are no specific effective antiviral medications or vaccines specifically designed to treat NiV infection. The WHO only recommends therapies for severe respiratory and neurological problems; therefore, prevention and intensive and supportive care will be the cornerstones of management [35]. Many antiviral medications were studied for the treatment of NiV, which are listed in *Table 1*.

Immunity Against NiV Infection

Immunity against the NiV has different characteristics in natural infections compared to experimental infections. The innate and adaptive immune response that develops in spontaneous infections has a significant impact on the onset of the NiV infection and its clinical prognosis. Interferon signaling, pro-inflammatory cytokine activation, and antibody synthesis are all involved in the immune response [45]. Viral glycoprotein antibodies are usually neutralized, resulting in long-term protective immunity in survivors. The kinetics of antibody production, cytokine expression, and T-cell activation can all be studied in experimental infection models, such as those in hamsters, ferrets, and non-human primates. Survival is linked to the production of neutralizing antibodies and virus-specific T-cell responses early in the infection. These immunological dynamics are crucial to understanding how to formulate effective vaccines and interventions against NiV [46].

The first defense against NiV infection is innate immunity, which occurs immediately upon viral entry before the adaptive immune system is stimulated. Neutrophils surround viral particles, which produce reactive oxygen species, antimicrobial peptides, and neutrophil extracellular traps (NETs), but excessive NET production causes tissue damage. NiV initially infects endothelial cells, epithelial cells, and selected immune cells upon natural infection, where it also induces recognition via pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) [47]. These receptors sense viral components, especially viral RNA, and cause the activation of signaling pathways, including NF-κB and IRF3/7, which promote type I interferons (IFN-α and IFN-β) production and pro-inflammatory cytokines. NiV infection stimulates production that is necessary in order to restrain viral replication. Important innate cytotoxic cells, including natural killer (NK) cells, recognize and kill NiV-infected cells by identifying stress-induced ligands and down-regulated MHC class I. Neutrophils and macrophages facilitate early viral clearance by phagocytosis and secretion of inflammatory mediators [48]. Nevertheless, in spite of these defenses, NiV contains a few structural and

Table 1. *Therapeutics evaluated against NiV*

Drug	Target	Mechanism of Action	Dose/Route	Key Findings	Advantages	Limitations	Study Type	Reference
Ribavirin	Humans/Syrian hamsters	Nucleoside analog; inhibits viral RNA synthesis	Oral/IV; early-phase administration	Reduced mortality in early human cases; limited effect if delayed	Moderate efficacy, Broad antiviral spectrum;	Weak evidence; toxicity at high doses	Retrospective/ *In vivo*	[36]
Remdesivir	African green monkeys	RNA-dependent RNA polymerase inhibitor	5-10 mg/kg, IV; pre- and post-exposure	Complete protection when given early; reduced viral load	High efficacy, Broad antiviral spectrum; already FDA-approved for other viruses	IV only; high cost	*In vivo*	[37]
Favipiravir	Syrian golden hamsters	Inhibits viral RNA polymerase, causing lethal mutagenesis	300 g/kg/day; within 24 h post-infection	Enhanced survival and reduced virus titer	Moderate to high efficacy, Oral bioavailability; Stockpiled for influenza	Teratogenicity; limited NiV human data	*In vivo*	[38]
Chloroquine	Vero E6 cells	Blocks endosomal acidification, interfering with viral entry	10-25 µM Within 24 h	Inhibited viral entry *in vitro;* lacked *in vivo* efficacy	Ineffective in vivo, Low cost; widely available	limited animal or clinical benefit	*In vitro*	[39]
Monoclonal antibody (m102.4)	Ferrets/African green monkeys	Neutralizes G glycoprotein, blocking viral attachment	15-50 mg/kg Single IV dose; 24-48 h post-exposure	Protected against disease progression and death	High efficacy, Strong preclinical/early human safety data	IV only; needs cold chain	*In vivo*	[40]
Monoclonal antibody cocktail (1F5 + m102.4)	African green monkeys	Broad neutralization of G glycoprotein variants	20 mg/kg IV combination; early post-exposure	Improved survivals and monotherapy; reduced viral load	Very high efficacy in animals may prevent escape mutants	High cost; logistical complexity	*In vivo*	[41]
Galidesivir (BCX4430)	Animal models (hamsters, NHPs)	Adenosine analog; inhibits viral RNA synthesis	50-100 mg/kg; early administration	Reduced mortality in filoviruses; NiV data suggest potential benefit	Broad-spectrum RNA virus activity	No human NiV trials	*In vivo*	[42]
Remdesivir + mAb combination	African green monkeys	Polymerase inhibition + viral neutralization	50 mg/kg IV combo; early post-exposure	Synergistic protection; improved survival	Very high efficacy, Combines two mechanisms; lowers resistance risk	Resource-heavy; IV	*In vivo*	[43]
Acyclovir	Vero cells	DNA polymerase inhibitor (herpesviruses)	10-50 µM	No significant antiviral effect against NiV	Ineffective for RNA viruses, Safe; widely available	Not active against RNA viruses	*In vitro*	[44]

non-structural proteins, including V, P, W, and M, which inhibit the IFN signaling by attacking the interaction between the proteins and the signaling transducer. IFN-I has been demonstrated to play a role in host defense against NiV infection in vivo in hamsters, ferrets, and mice, and interventions that drive IFN-I signaling, such as poly(I)-poly(C12U), can improve survival. Excessive release of pro-inflammatory cytokines, such as TNF-α and IL-1β, is also involved in the neurological pathology and disrupts the blood-brain barrier [49].

Adaptive immunity is very significant in the control and clearance of NiV infection, which is observed following the primary innate reaction. B lymphocytes generate virus-specific antibodies and memory B cells to produce humoral immunity against NiV to provide both long and short-term protection. After the first exposure, IgM antibodies are produced, and then IgG, IgA, and other immunoglobulin subclasses are also produced. And when re-exposed, there is a fast, robust response by means of memory B cells [50]. There is a limited amount of human data, although a 2018 NiV outbreak in India involving human subjects reported that survivors produced NiV-specific IgM and IgG antibodies, with an increase in the number of B cells, which is an indication that adaptive immunity has worked. In animal experiments, it has been further shown that neutralization antibodies are produced in 1-2 weeks post-infection in swine, although viral RNA may remain in the presence of antibodies [51]. Late B-cell response was observed to be linked with the fast disease progression in African green monkeys, and the early production of IgM and IgG correlated with the survival of monkeys. These findings collectively indicate that humoral responses are of great importance in viral clearance and protection, but the kinetics and magnitude of B-cell and antibody responses vary across species and define disease outcome [52].

T lymphocytes are the cellular immune mediators that play a crucial role in the control and removal of NiV infection. The coordinating activity of T cells is that CD8+ cytotoxic T cells identify viral peptides displayed on MHC class I molecules, killing infected cells directly by the process of apoptosis and cytolysis, whilst CD4+ helper T cells promote B-cell activation and cytotoxic effects by secreting cytokines. The expansion and functioning of these effector cells are coordinated by cytokines like IFN-γ, TNF-α, and IL-2. The adaptive response in the memory B and T cells induces long-lasting immunity, allowing rapid and intense reactions to a new exposure [53]. Animal models, including African green monkeys, swine, mice, and others, were involved in the process of virus clearance accompanied by enhanced production of cytokines and chemokines, which supported the role of both CD4+ and CD8+ effector and memory T cells in the process. Mice vaccine experiments demonstrated that NiV-specific T cell responses could be induced against F and G protein epitopes, suggesting potential vaccine targets. In addition, T cells that had cross-reacted during earlier exposure to other *Paramyxoviridae* viruses, such as measles and human parainfluenza, were able to recognize and kill NiV-infected cells, which indicated that cellular immunity can be more broad-based in terms of protection against antigenically related viruses [5].

Vaccine Development

NiV is a life-threatening disease because it is widely distributed and has no effective cure. Preventing the NiV infections to reduce the spread, through the development of vaccines, would help relieve it among vulnerable populations. But making vaccines against the reservoir of the virus, the bats, is very impractical since working with a live pathogen may cause biohazards and other possible issues of administration. The immunogenic agents of the traditional vaccine are usually weakened pathogen or their proteins, but other types of antigens are more favoured by the investigators due to the fear of biohazards [54]. An interesting approach is through subunit vaccines, which use fragments of protein or a glycoprotein of a pathogen and produce a protective immune reaction to it, e.g., in the cat family, soluble G glycoprotein, when administered subcutaneously, alone is capable of inducing serum-neutralizing antibodies. In cats that were kept vaccinated, the levels of antibody were significantly higher (titer ~20,000) up to 2 months [55].

The development of the NiV vaccine has been done in various approaches, and the surface glycoproteins have formed a major consideration, G-glycoprotein and F-glycoprotein. The former involves a recombinant subunit vaccine such as Hendra virus subunit glycoprotein (HeVsG) that has been shown to offer protection against NiV and HeV challenge on ferrets, African green monkeys (AGM), as well as rabbits. Applied in Australia to horses (Equivac HeV, Zoetis) as a veterinary vaccine against HeV. The HeVsG is also being evaluated as a possible human vaccination against NiV. Thus, the immunization of ferrets by the HeVsG demonstrated exceptional outcomes with respect to disease prevention and a reduction in NiV replication and protected the ferrets for at least 14 months. Nonetheless, HeVsG vaccination induces cross-neutralising antibodies against NiV in pigs, though they were not at protective levels [56]. The concentrations of cross-neutralizing antibodies in these animals rose about 80-fold after 5-7 days after making the challenge; however, they did not have significant cellular immunological memory and protection. It is interesting to mention that pigs that were exposed to NiV orally and nasally produced a protective antibody response and cell-mediated immune (CMI) memory response. The following are

viral recombinant vectors that have been demonstrated to protect hamsters, pigs, ferrets, and/or AGMs against NiV challenge: recombinant *Rhabdoviruses* (VSV and rabies) expressing NiV F or NiV G, vaccinia viruses encoding both NiV F and NiV G, canarypox encoding NiV F or NiV G, and recombinant measles virus vector expressing NiV G. In spite of the fact that antibodies against the G or F glycoproteins may lead to neutralization of viruses, G appears to be the most prevalent and co-dominant target of neutralizing antibodies [57].

Integrating One Health in NiV Surveillance

It is important that the One Health strategy be included in NiV surveillance in order to manage this new zoonotic threat in a coherent way. This plan unites communities, domains, and sectors across various levels of society to work together in the prevention, forecasting, detection, and response of global health emergencies such as pandemics. Given that the One Health strategy takes into consideration the aspects of the environment, veterinary health, and public health, it can be deployed to improve the monitoring of this virus and minimize the chances of severe NiV outbreaks. Health-based surveillance involves coordinated efforts by veterinarians, animal biologists, environmental scientists, and medical professionals to detect and follow the virus throughout its several reservoirs and transmission pathways [58]. High-risk areas, where agricultural activities, ecological disturbance, and human-wildlife contact raise the possibility of spillover occurrences, should be given priority by surveillance systems. Predictive skills can be further improved by environmental monitoring, which includes land-use changes and climatic variability.

Future Perspective and Conclusions

Most countries do not have specialized surveillance tools, diagnostic instruments, or emergency measures that plan to fight this virus, implying that the world is unprepared for a Nipah pandemic. Even though the WHO and other agencies have classified NiV as a priority research and development pathogen, it is imperative to note that there is an urgent need for more funds to be allocated to monitoring, educating the masses, developing vaccines, thus a possible health emergency in the future can be prevented. In the last decades, particular attention was given to the pathophysiology of the NiV and its transmission method. This knowledge was improved in the next decades. Here, it is important to keep in mind that the prudent application of the same knowledge is needed so that the NiV vaccine can be developed, subject to human clinical trials, and risk factors can be reduced to prevent the occurrence of infection. This zoonotic illness must be prevented at all costs [59]. Given the necessity for better communication between veterinary and medical services on the illness, scientists have established a global outbreak network and a response network, particularly in the wake of the outbreaks in Bangladesh and India. Some strong preventative measures may be created and put into action by incorporating many industries and multi-sector approaches. NiV has a high zoonotic effect and a high mortality rate. Frequent NiV outbreaks have resulted in several human and animal deaths and morbidities over the past 20 years, despite multiple warnings. Furthermore, because of its high mortality rate and propensity to cause a major physical and financial burden, prevention of this illness is crucial owing to its pandemic potential. In order to stop any more NiV outbreaks, health officials must immediately begin clinical trials to determine potential treatment plans.

Declarations

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RESEARCH ARTICLE

Effects of Probiotics (*Bacillus subtilis*), Prebiotics (MOS + β-Glucans) and Their Combination on Growth Performance, Duodenal Histomorphology and Meat Quality in Broiler Chickens

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Abstract

The aim of this study was to evaluate the effects of dietary supplementation with probiotics, prebiotics, and synbiotics on growth performance, carcass traits, meat quality, and histomorphological characteristics in broiler chickens. A total of 320 one day old broiler chicks were allocated to four dietary treatment groups, each comprising 8 replicates of 10 chicks in completely randomized design. The groups included: a control group (no supplement), a prebiotic group (1 g/kg β-glucan + mannan-oligosaccharide; BM), a probiotic group (1 g/kg *Bacillus subtilis*; BS), and a synbiotic group (0.5 g/kg β-glucan + mannan-oligosaccharide + 0.5 g/kg *Bacillus subtilis*; BM + BS). The results revealed that body weight (BW), body weight gain (BWG), and average daily feed intake (ADFI) significantly increased in the BM + BS group, while feed conversion ratio (FCR) improved compared to the control group ($P<0.001$). Furthermore, slaughter weight, hot and cold carcass weights, as well as heart and gizzard weights, were significantly higher in the BM + BS and BS groups ($P<0.05$). Histomorphological analysis showed that villus height to crypt depth ratio (V/C) was significantly greater in the BM + BS and BS groups, but lower in the BM group ($P<0.001$). Regarding meat quality, the BM + BS and BS groups showed increased brightness (L*), redness (a*), and yellowness (b*) values ($P<0.05$). In conclusion, the dietary synbiotics supplementation in broiler diets was shown to enhance growth performance, improve intestinal morphology.

Keywords: Growth performance, broilers, prebiotics, probiotics, synbiotics

INTRODUCTION

The extensive use of antibiotics in animal husbandry has contributed to resistant strains of bacteria and antibiotic residues in meat, causing serious health risks for human beings and the environment [1]. This has prompted a move from antibiotic growth promoters to developing alternative measures. It has been made possible for modern poultry farmers to rear chickens to the slaughter weight in a short time because of advancements made in chicken genetics and feeding techniques [2]. Nevertheless, the rapid growth rates of these broiler strains are accompanied by greater vulnerability to stressors, which can impair growth efficiency and ultimately compromise production outcomes [3]. Moreover, stress leads to pronounced biochemical and physiological alterations in the animals, resulting in antioxidant depletion, hence the degradation of meat quality [4].

As a result, meeting higher consumer expectations and improving meat quality has created a need for new approaches to animal nutrition [5]. In this regard, researchers aiming at replacing antibiotics with natural additives like probiotics, prebiotics, and synbiotics have gotten traction [6-9]. Probiotics, in particular, have attracted substantial scientific interest since the pioneering studies on replacing antibiotics with live microorganisms in poultry [10]. They are defined as a selective mixture of microorganisms primarily *Lactobacilli, Streptococci*, and *Bacillus* species that support intestinal health by modulating the gut flora through antagonism against pathogenic bacteria [11-13].

Prebiotics are substrates selectively metabolized by gut microorganisms to confer health benefits to the host [14]. Probiotics and prebiotics improve intestinal health, control

foodborne pathogens, and strengthen the immune system [15,16]. These practices modify the composition of the gut microbiota by augmenting the populations of useful bacteria *(Bifidobacteria* and *Lactobacilli)* and decreasing harmful bacteria *(E. coli* and *Campylobacter)* [17]. Moreover, the results suggest improvement in gut structure, serum immunological responses, and production of short chain fatty acids [18].

The combined use of probiotics and prebiotics is referred to as a synbiotic approach [19]. Prebiotics bind to the fimbriae of the harmful bacteria, facilitating their removal via the fecal bolus while simultaneously promoting the growth and metabolism of beneficial microorganisms. Additionally, probiotics enhance enterocyte nutrition, stimulating the digestive system and promoting intestinal balance and health in birds [20].

The study aimed to determine the effects of dietary supplementation with probiotics, prebiotics, and synbiotics on growth performance, intestinal health, and meat quality in broilers. Although several studies have been conducted to evaluate the effects of probiotics, and prebiotics on broiler performance, gut health and serum biochemical parameters. However, results have been inconsistent and information on the the the synbiotic effects of Bacillus subtilis with β-glucan and mannan-oligosaccharide on both intestinal histomorphology and meat quality is limited. Therefore, this study hypothesized that probiotic and prebiotic individually and synbiotic supplementation would synergistically improve growth performance, duodenal histomorphology, and meat quality characteristics in broiler chickens.

Material and Methods

Ethics Statement

All methods employed in this study were conducted following the guidelines approved by the Kafkas University Ethics Committee (KAÜ-HADYEK/2024-128).

Experimental Birds, Husbandry, and Diets

The experiment was carried out at the Broiler Unit, Faculty of Veterinary Medicine, Kafkas University. Two sources of supplementation included beta-glucan + mannan-oligosaccharide (Vimar Company, Türkiye) and *Bacillus subtilis* (Kartal Kimya, Türkiye). A total of 320 one day-old mixed-sex Ross 308 broiler chicks were obtained from a local commercial producer, weighed, and assigned to four different dietary treatment groups in a completely randomized design, with eight replicates of 10 chicks each (initial weight: 44.66±0.08 g). The chicks were housed in floor pens with dimensions of 130 cm in width, 108 cm in length, and 54 cm in height. The temperature control started with a reduction from an initial temperature of 35°C on day one, decreasing gradually at a rate of 0.5°C per day until a temperature of 26°C, from which point it remained steady at that temperature until day 42. During the experiment, the average relative humidity ranged from 60% to 75%, and a lighting regimen of 23 hours of light and 1 hour of darkness was applied until day 42. The broilers were fed according to a two-phase feeding program, consisting of a starter diet (0-21 days) and a finisher diet (21-42 days). The control group received no dietary supplementation, whereas the experimental groups were fed as follows: basal diet + 1 g/kg prebiotic (β-glucan + mannan-oligosaccharide [BM]); basal diet + 1 g/kg probiotic *(Bacillus subtilis* [BS]); basal diet + 0.5 g/kg prebiotic + 0.5 g/kg probiotic (BM + BS) synbiotic group. Prebiotic (ß-glucans and mannanoligosaccharides) and probiotic BS supplements were sourced from a commercial supplier. The base diets for each phase were formulated according to the nutritional requirements of Ross 308 broiler chickens, as defined by the NRC *(Table 1)* [21]. Prebiotic and probiotic premixes were manually incorporated into the mash feed. All experimental groups were provided with powdered feed, and drinking water was available *ad libitum* throughout the study.

Growth Performance and Organ Index

Birds were individually weighed using a digital weighing scale on days 1, 21, and 42. Body weight gain (BWG) was calculated using a differential method. Feed intake (FI) was determined on intervals by measuring the difference between unconsumed and offered feed. Feed conversion ratio (FCR) was calculated by dividing FI by BWG. Adjustments were made to the BWG, FI, and FCR calculations to account for mortality and to ensure accurate data representation [22]. On day 42 of the study, one broiler chicken per cage was randomly selected from each of the four experimental groups (eight birds per group) and sacrificed by cervical dislocation. Defeathering was performed with the hard scaling process as described by Shung et al. [23]. Slaughter weight and hot carcass weight were recorded (g). The weight of internal organs (heart, liver, gizzard and spleen) was recorded. Cold carcass weight was measured after being stored at 4°C for 24 h. Hot and cold carcass yields were determined based on recorded carcass weights.

Intestinal Relative Index and Histomorphological Analysis

On day 42 of the study, mid-segment duodenal samples (approximately 2 cm in length) were collected from eight birds per treatment group (the same birds used for carcass evaluation, one per cage) and preserved in 10% buffered formalin for fixation. Following fixation, the samples were washed, dehydrated, cleared, and paraffin embedded. Sections of 5 µm thickness were cut out of the

Table 1. Ingredients and nutrient composition of the basal diet for broilers chicken (g/kg of diet on an as-fed basis, at 90% DM)

Ingredients (%)	Starter	Finisher
Corn, yellow	30.00	46.10
Barley	10.00	7.20
Wheat	5.70	8.00
Bran	2.80	2.50
Wheat middlings	3.50	2.40
Wheat offal	10.00	2.00
Vegetable oil	3.10	3.80
Rice bran	2.00	2.00
Sunflower meal, 45% CP	4.55	5.50
Corn gluten meal, 62% CP	11.00	10.25
Soybean meal, 48% CP	13.00	6.50
Dicalcium phosphate	1.80	1.40
DL-methionine	0.25	0.20
L-Lysine	0.50	0.50
Threonine	0.25	0.20
Marble dust	1.15	1.00
Salt	0.20	0.25
Vitamin-mineral premix[1]	0.20	0.20
Nutrients levels2 (%)		
Metabolizable energy, kcal/kg	3001	3201
Dry matter	89.90	90,00
Crude protein	22.50	19.50
Phosphorus	0.48	0.38
Calcium	0.95	0.78
Methionine +Cystine	1.04	0.92
Lysine	1.16	0.99
Ether extract	5.44	6.20
Crude fiber	4.26	3.73
Ash	6.02	5.11

1 Supplied per kilogram of diet: 1.537.200 mg vit. A, 6.28 mg vit. E, 0.64 mg vit. K3, 37.36 mg Mn, 25 mg Zn 89, mg Fe, 0.03 mg Co, 8.76 mg Cu, 0.05 mg Mg, 0.91 mg Se
2 Calculated compositions.

paraffin blocks and stained with Hematoxylin and Eosin for histomorphometric analysis of villi length and crypt depth *(Fig. 1)*. Measurements were manually conducted on an area of 30.000 µm (the size of 20 fields of view) using the Cameram SLR 6.1 software for digital analysis (Mikro Sistem Ltd., Türkiye), and the corresponding arithmetic means were computed. The villus height to crypt ratio (V/C) was calculated [20].

Meat pH Value

At 15 min and 24 h after slaughter, pH values of the breast muscles were measured at a depth of 2.5 cm

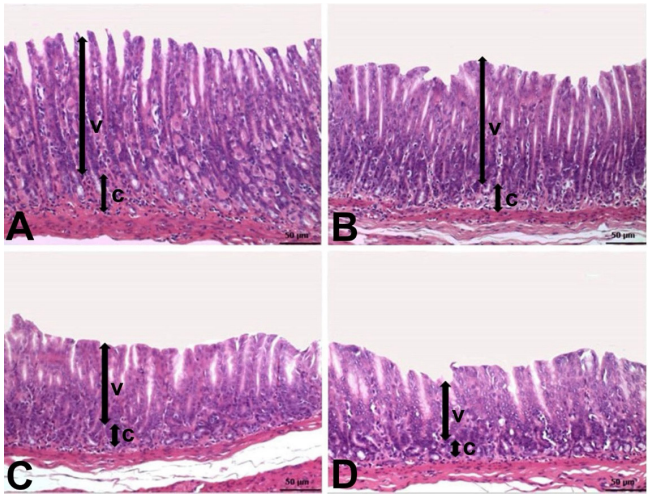

Fig 1. Broiler duodenum, Control: basal diet; BM: 1 g/kg prebiotic powder (β-glucan + mannanoligosaccharide), BS:1 g/kg probiotic powder (Bacillus subtilus), BM+BS: 0.5 g/kg probiotic (Bacillus subtilus) + 0.5 g/kg prebiotic (β-glucan + mannanoligosaccharide), V = villus height, C = Crypt depth. H&E staining

below the surface from three different points a combined glass penetrating electrode (Hanna instruments, Inc. Woonsocket, USA) [24].

Meat Color

Color measurements were taken on the carcass surface of the breast muscles, as well as the freshly exposed cut surface of the muscle. The L* (lightness), a* (redness), and b* (yellowness) values were determined using a chromameter (Hangzhou CHNSpec Technology Co., Ltd., China) [24].

Data Analysis

All the experimental data were processed using SPSS (PASW Statistics 22) software. One way ANOVA followed by Tukey's multiple comparisons were used to assess the effects of dietary treatments on the measured values. Differences were considered significant at $P<0.05$.

Results

Growth Performance

Dietary supplementation with prebiotics, probiotics, and synbiotics significantly ($P<0.005$) improved body weight and body weight gain compared to the control group *(Table 2)*. The most notable improvement was observed in the BM + BS group, followed by the BS and BM group. Birds receiving the BM + BS had higher average daily feed intake (ADFI), while the control, BM, and BS groups exhibited similar ADFI values (21-42 d and 0-42 d). The BM + BS group observed the best FCR during days 0-21, 21-42, and across the entire study period (0-42 days) ($P<0.001$). Additionally, the BS group showed improved FCR compared to the control group, whereas the BM was similar to the control group but exhibited a tendency toward improvement.

Carcass parameters

As shown in Table 3, dietary inclusion of BS and BM + BS resulted in a significant improvement in slaughter, hot carcass, and cold carcass weights relative to the control group (P<0.001). Improvements were observed in the BM+BS group than in the BS group (P<0.001). Both BS and BM + BS feeding also resulted in significant increases in heart weight and gizzard weight. No significant variations were seen in liver weight, spleen weight, or in the percentages of hot and cold carcass yield among groups (P>0.05).

Intestinal Histomorphology

As presented in *Fig. 2*, the V/C ratio was significantly increased by BS and BM + BS supplementation, whereas BM addition resulted in a significant decrease (P<0.001). Interestingly, despite the increase in the V/C ratio, the

Table 2. The effect of dietary supplementation with prebiotics, probiotics, and synbiotics on the performance parameters of broilers

Item	Dietary Treatments1				P-value
	Control	BM	BS	BM+BS	
d 1-21					
BW (g/bird)	350.89±2.41^{c}	365.62±2.86^{b}	373.68±2.29^{ab}	385.62±4.62^{a}	0.001
BWG (g/bird/ d)	28.12±0.22^{b}	28.96±0.43^{ab}	29.7±0.24^{ab}	30.61±0.75^{a}	0.005
ADFI (g/bird/ d)	38.94±0.31	39.41±0.58	39.65±0.19	40.04±1.05	0.659
FCR	1.36±0.01^{c}	1.34±0.01^{bc}	1.32±0.01^{ab}	1.3±0.01^{a}	0.001
d 22-42					
BW (g/bird)	1714.5±5.95^{d}	1809.92±12.23^{c}	1863.9±15.93^{b}	1990.58±13.07^{a}	0.001
BWG (g/ d)	81.35±0.33^{c}	84.87±1.01^{bc}	87.32±1.50^{b}	93.38±1.33^{s}	0.001
ADFI (g/bird/ d)	125.5±1.03^{b}	127.62±1.3^{b}	129.61±2.35^{ab}	136.38±0.68^{a}	0.001
FCR	1.53±0.01^{b}	1.5±0.01^{ab}	1.48±0.02^{ab}	1.46±0.0^{2a}	0.032
d 1-42					
BW (g/bird)	1032.69±2.48^{d}	1087.77±5.78^{c}	1118.79±7.41^{b}	1188.10±7.59^{a}	0.001
BWG (g/ d)	54.73±0.12^{c}	56.92±0.33^{b}	58.51±0.70^{b}	61.99±0.45^{a}	0.001
ADFI (g/bird/ d)	82.22±0.48^{b}	83.52±0.56^{b}	84.63±1.17^{b}	88.21±0.46^{a}	0.001
FCR	1.45±0.01^{c}	1.42±0.01^{bc}	1.40±0.01^{ab}	1.38±0.01^{a}	0.001

BWG: body weight gain; BW: body weight; FI: feed intake; FCR: feed conversion ratio
a,b,c,d Each superscript indicates the difference between the means within the row (P<0.05)
1 Control: basal diet without supplementation; BM: Basal diet supplemented with 1 g/kg prebiotic powder (β-glucan + mannanoligosaccharide), BS: Basal diet supplemented with 1 g/kg probiotic powder (Bacillus subtilis), BM+BS: Basal diet supplemented with 0.5 g/kg probiotic (Bacillus subtilis) + 0.5 g/kg prebiotic (β-glucan + mannanoligosaccharide)

Table 3. The effect of dietary supplementation with prebiotics, probiotics, and synbiotics on some carcass parameters in broilers

Item (g)	Treatments[1]				P-value
	Control	BM	BS	BM+BS	
Slaughter weight	2380.99±41.76^{c}	2443.11±27.99^{bc}	2521.61±19.04^{b}	2636.92±21.06^{a}	0.001
Hot carcass weight	1758.00±31.40^{c}	1802.67±22.57^{bc}	1863.33±14.41^{b}	1950.17±16.03^{a}	0.001
Cold carcass weight	1743.00±31.40^{c}	1787.67±22.57^{bc}	1848.33±14.41^{b}	1935.17±16.03^{a}	0.001
Hot carcass yield %	73.83±0.14	73.77±0.16	73.89±0.02	73.95±0.02	0.679
Cold carcass yield %	73.20±0.14	73.16±0.17	73.30±0.02	73.38±0.02	0.474
Liver	58.19±1.06	54.48±2.69	56.29±1.31	52.56±1.43	0.137
Gizzard	24.62±0.54^{b}	24.75±1.29^{b}	26.33±0.64^{ab}	29.10±1.02^{a}	0.030
Heart	12.99±0.37^{b}	14.65±0.30^{ab}	15.48±0.49^{a}	15.02±0.69^{a}	0.004
Spleen	4.05±0.35	3.69±0.14	3.46±0.10	4.10±0.18	0.110

a,b Each superscript indicates the difference between the means within the row (P<0.05)
1 Control: basal diet without supplementation; BM: Basal diet supplemented with 1 g/kg prebiotic powder (β-glucan + mannanoligosaccharide), BS: Basal diet supplemented with 1 g/kg probiotic powder (Bacillus subtilis), BS+BM: Basal diet supplemented with 0.5 g/kg prebiotic (β-glucan + mannanoligosaccharide) + 0.5 g/kg probiotic (Bacillus subtilis)

supplementation of BM, BS and BM+BS in the diet significantly (P<0.001) reduced both villus height and crypt depth in broilers *(Fig. 2)*.

Meat Quality

The impacts of prebiotic, probiotic and synbiotic supplementation on muscle pH and meat color values are presented in *Table 4.* All meat quality traits, including breast color, differed significantly among experimental group (P<0.001). Birds treated with BM + BS and BS had lighter breast meat compared to the control group, while their breast color was similar to that of the BM group. The redness (a*) of the meat was significantly higher in the BM + BS group compared to the control group. The BS group had the highest redness, followed by the BM group (P<0.001). The BM + BS group showed higher yellowness (b*) compared to the control group and BM treated birds, while the BS treated birds had similar yellowness to all other group (control, BM and BM + BS). The addition of BM, BS and BM + BS significantly increased the pH of the pectoral muscle compared to the control group (P<0.001).

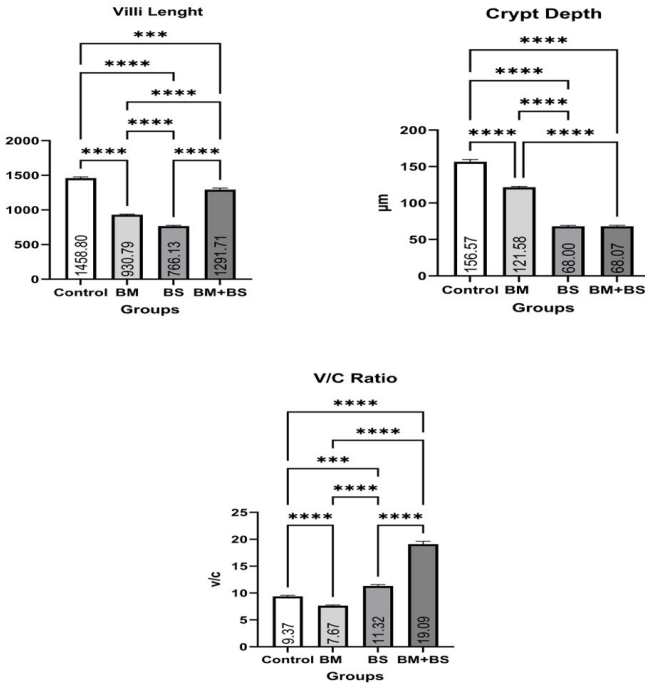

Fig 2. Impact of prebiotic, probiotic, and synbiotics supplementation on villus height, and villus height /crypt depth ratio. V/C: villus villus height/crypt depth. The X-axis displays villus length, crypt depth, and V/C ratio (µm), while the Y-axis shows their distribution in the control and experimental groups. Control: basal diet; BM: 1 g/kg prebiotic powder (β-glucan + mannanoligosaccharide), BS:1 g/kg probiotic powder (Bacillus subtilus), BM+BS: 0.5 g/kg probiotic (Bacillus subtilus) + 0.5 g/kg prebiotic (β-glucan + mannanoligosaccharide) Error bars indicate standard deviation. Control and other groups: ***P<0.05, ****P< 0.01

Discussion

Studies have demonstrated that prebiotics, probiotics, and synbiotics have a positive correlation with increased weight gain, FI, and FCR in broilers [25-27]. Our findings demonstrate that prebiotic and probiotic supplementation improved performance parameters, with synergistic increase in the synbiotic group. Our results corroborate previously established research, supporting the claim that supplementation with prebiotics, probiotics, and synbiotics has a positive correlation with broiler performance, particularly in the synbiotic supplemented group, which showed the highest response [28,29]. Conversely, Sahin et al.[19] observed that the administration of prebiotic and probiotic supplements did not yield any statistically significant impact on body weight gain and feed conversion ratio. Furthermore, feed intake increased in the synbiotic group, while there were no changes in the prebiotic and probiotic treatments. These findings are in accordance with Abdel-Fattah and Fararh [28] who did not find an effect of probiotic and prebiotic supplementations on feed intake. While the impacts of synbiotic supplementation on the growth performance of broilers are generally positive. The result depends on the type of synbiotic, the application method, and the chicken's genotype [30-32]. Our findings indicated that the FCR improved in both synbiotic and probiotic treatment groups, with the best improvement in the synbiotic group. These findings are in accordance with

Table 4. Effect of probiotic, prebiotic, and synbiotics supplementation on broiler meat quality

Item	Treatments[1]				P-value
	Control	BM	BS	BM+BS	
pH**15min**	5.96±0.03^{b}	6.32±0.06^{a}	6.25±0.04^{a}	6.27±0.06^{a}	0.001
pH**24h**	5.75±0.03^{b}	6.11±0.07^{a}	6.05±0.04^{a}	6.07±0.06^{a}	0.001
Lightness (L*)	44.28±0.67^{a}	42.97±0.81^{ab}	40.45±0.55^{b}	40.77±0.68^{b}	0.001
Redness (a*)	1.98±0.12^{b}	2.50±0.26^{ab}	2.70±0.31^{ab}	3.19±0.26^{a}	0.001
Yellowness (b*)	10.13±0.32^{b}	10.34±0.70^{b}	11.23±0.38^{ab}	12.21±0.52^{a}	0.018

a,b Each superscript indicates the difference between the means within the row (P<0.05)
1Control: basal diet without supplementation; BM: Basal diet supplemented with 1 g/kg prebiotic powder (β-glucan + mannanoligosaccharide), BS: Basal diet supplemented with 1 g/kg probiotic powder (Bacillus subtilis), BM+BS: Basal diet supplemented with 0.5 g/kg prebiotic (β-glucan + mannanoligosaccharide) + 0.5 g/kg probiotic (Bacillus subtilis)

Abdel-Fattah and Fararh [28] who reported the low FCR in the synbiotic treatment group, followed by the probiotic treatment group. These improved performance indices that arise from synbiotic supplementation are likely the result of the stimulation of beneficial gut microbials. This synergistic effect stems from enhanced gut microbial fermentation and host nutrient utilization [33]. With the prospects of improving growth performance, synbiotics are a promising alternative to antibiotics [34].

Our study demonstrated that the synbiotic and probiotic groups achieved the highest slaughter weight, hot carcass weight, and cold carcass weight, respectively. Previous studies have also found that synbiotics positively affect carcass characteristics in broilers [28,35,36]. Cheng et al.[25] reported no synbiotic effect on carcass weight, although some studies have reported improvements in breast yield in the synbiotic group [25,28,37]. In our study, there were no significant differences in the percentages of cold and hot carcass, gizzard weight, or liver weight, consistent with the work by Sarangi et al.[16] In addition, Chumpawadee et al.[38] reported no probiotic effect on these parameters. Notably, gizzard weight was significantly higher in the synbiotic group, and the maximum heart weight was in the probiotic and synbiotic groups, consistent with the work by Tayeri et al.[39] who reported increased heart weight after synbiotic supplementation. This increased gizzard and heart weights can be attributed to the improved gut functionality and metabolic activity of the broiler chickens. Gizzard play a major role in the mechanical digestion of feed. The synergistic effect of the probiotic and prebiotic promoted higher FI through gut microflora and enzymes modulation, resulting in prolonged retention time of the feed in the upper digestive tract and greater muscle development of the gizzard for improved gastrointestinal functioning [40]. The increased heart weight in the synbiotic group likely indicates improved metabolic performance and the associated increase in circulatory demand. Birds in the supplemented groups showed higher FI, ADG and improved FCR and this improvement were highest in the synbiotic supplemented group. Birds exhibiting superior growth and feed efficiency require increased oxygen and nutrient supply, which may stimulate the cardiac muscle development as a physiological response.

Intestinal integrity and function in broilers are greatly affected by villus structure, specifically villus length and crypt depth, which characteristically regulate the capacity to absorb nutrients. A greater V/C ratio is highly correlated with increased intestinal integrity and efficiency of nutrient utilization [41]. Increased villus height enhances feed efficiency by increasing the area for nutrient uptake [42]. Synbiotics, probiotics, and prebiotics have been reported to increase villus length and the V/C ratio, mainly by reducing crypt depth [43]. The intestinal crypts are the places at the base of the villi where stem cells proliferate to replace the enterocytes. A shallow crypt indicated longer lifespan and a slower turnover rate of epithelial cells. Thus, lower crypt depth is considered as a positive indicator of intestinal health and functional maturity. In the present study, the addition of probiotic, prebiotic, and synbiotic supplements also resulted in the reduction in the depth of the crypts following the previous trend. Contrary to previous reports, the present study noted a decline in villus length with supplementation with these additives. Therefore, villus height alone is insufficient for evaluating the effects of probiotics, prebiotics, and synbiotics on intestinal health; it must be considered alongside other intestinal morphology and performance parameters. Extensive studies have shown that dietary supplementation with probiotics and synbiotics improves intestinal morphology as seen by significant increments in the V/C ratio [44,45]. In this study, the V/C ratio increased in the probiotic and synbiotic treatment groups. The performance improvements observed can be attributed to the ability of biological feed additives to modulate microbial populations and gastrointestinal pH, thereby enhancing nutrient absorption and feed utilization efficiency [46]. In our study, the V/C ratio was lowered in the prebiotic group. Although prebiotics generally enhance the V/C ratio [45,47], exceptions can occur depending on specific conditions. This underscores the fact that the effects of prebiotics and probiotics can vary based on species, dosage, and application methods. These findings suggest that probiotics, prebiotics, and synbiotics optimize gut health and mucosal efficiency rather than just only increasing villus height.

Meat color is the first sensory trait that the customer perceives and the most significant factor in product acceptance or rejection since meat color often relates to other quality factors such as freshness, nutritional value, maturity, or spoilage [48]. Modifying the dietary pattern has been the strategic approach towards meat quality improvement, particularly for broilers [24]. Myoglobin content and muscle tissue pH are two significant factors that determine meat color and color defects [49]. The L* value indicates meat brightness, with higher values representing paler colors, while higher a* and b* values reflect consumer desirable redness (freshness) and healthier pigmentation, respectively [50]. In this study, probiotic and synbiotic supplementation reduced breast muscle L* values (darker color) while increasing a* (redness) across all treated groups. Synbiotics further enhanced b* (yellowness) compared to controls. Consistent with the findings of the present study, it has also been reported that supplementation with *B. subtilis* increased the a* and b* values of meat color [4,24]. These changes in the meat color parameters can be explained

by the modulatory effects of the probiotic, prebiotic and synbiotic on the gut health, antioxidant status and muscle metabolism. The probiotic and prebiotic supplementation enhances the intestinal integrity and nutrient absorption including pigments such as carotenoids and xanthophylls that contribute to b* values of the meat [51]. moreover, improved a* values of meat color can be attributed to the higher blood circulation, antioxidant enzyme activity, and myoglobin stability. Probiotic, prebiotics and synbiotics are known to decrease the antioxidant levels, reducing lipid peroxidation in the muscle tissue, and prevent oxidation of myoglobin in the muscles resulting in a higher a* values of meat [52]. The lower lightness in the supplemented groups suggests that meat retained better muscle pigment integrity that resulted in darker and more natural color. Tavaniello et al.[53] reported decreased a* values following prebiotic supplementation in broilers, whereas conflicting results were observed by other studies, which reported increased L* values [4, 24]. However, no differences among meat color parameters were observed in some studies [54-56].

Meat quality is based to a great extent on the pH level of the rigor mortis process that encompasses the biochemical reactions occurring as the muscle tissue is converted to meat following slaughter [49]. In the current research, the pH level of the meat was significantly higher in the prebiotic, probiotic, and synbiotic supplemented groups. Meat pH findings are extremely variable in the previously reported literature. While there have been researchers who have observed the trend to be decreasing [4,24], some researchers observed the trend to be increasing [55], and a few have observed no significant impact [54,56]. In broilers, muscles with pH values above 6.0 contain minimal protein denaturation that manifests as low light scattering and translucency. In contrast, muscles with pH values below 6.0 contain increased protein denaturation that causes opaque appearance and increased light scattering [49]. Optical properties of meat that depend on pH influence the light reflected by internal and external surfaces. Light scattering has minimal influence on color properties such as redness (a*) and yellowness (b*) but has large impacts on meat brightness (L*) and pigment concentration [57]. Low pH poultry meat has also been associated with low water holding capacity that causes increased shelf life but reduced tenderness [58].

This research tested the impact of probiotic, prebiotic, and synbiotic supplementation on broiler growth performance, meat quality, and intestinal health. Results indicated that probiotic, prebiotic, and synbiotic supplementation improved growth performance in the broilers, with maximum improvement observed in the synbiotic groups. Generally, synbiotic supplementation resulted in higher increases in BW, BWG, and ADFI, as well as improving the feed conversion ratio. Intestinal morphology, as measured in terms of the V/C ratio, improved in the probiotic group as well as in the synbiotic group. In addition, breast meat color and pH was high in prebiotic, probiotic, and synbiotic supplemented broilers relative to the unsupplemented group. In conclusion, based on the findings, synbiotic supplementation can be an effective management system for improving broiler growth performance, intestinal health, as well as increasing the quality of the meat, in meeting the increasing demand for high quality chicken meats.

Declarations

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Research Article

Supplemented Pulsatilla Decoction Alleviated Ulcerative Colitis by Attenuating the Pro-Inflammatory Response and Modifying Gut Microbiota in Mice

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Abstract

The present study aimed to combine *Portulaca oleracea* L with PD to compose a new formula supplemented PD (SPD), and evaluate its effect on ulcerative colitis (UC). The UC mouse model was obtained by supplementing 3.5% dextran sulfate sodium salt (DSS) orally. Subsequently, the mice were treated with PBS, sulfasalazine, high dose SPD, medium dose SPD or low dose SPD. Morphological analysis, RT-qPCR, ELISA assay, LC-MS/MS analysis and 16s rRNA sequencing were conducted to evaluate the effect of SPD on UC. DSS treatment resulted in the decreased body weight and increased DAI value of the mice, while SPD mitigated the changes of body weight and DAI value induced by UC. SPD attenuated the proinflammatory response induced by UC by down regulating the expression of proinflammatory cytokines, and alleviated the oxidative stress in the gut of the mice with UC by downregulating the MDA level and enhancing the activity of antioxidant enzymes. Additionally, SPD also enhanced the transcription of *MUC2* and *Occludin* which were inhibited by UC. Furthermore, DSS induced the dysbiosis of the colonic microbiota and relative abundance of the *Akkermansia* at genus level was higher in UC group whereas treatment with high-dose SPD restored its abundance to levels comparable to the control group. SPD demonstrated therapeutic effects on DSS-induced UC of the mice by attenuating the proinflammatory response, enhancing the antioxidant ability and key molecules related with barrier function, and modifying the gut microbiota, thereby providing a potential strategy for the treatment of ulcerative colitis.

Keywords: Gut microbiota, Supplemented pulsatilla decoction, Ulcerative colitis, Proinflammatory response

Introduction

Ulcerative colitis (UC), a subtype of inflammatory bowel disease (IBD), is characterized by chronic, recurrent, and non-specific inflammatory lesion in the mucosa of large intestine [1,2]. As a relapsing and remitting disorder, UC not only impairs the absorption of the nutrients, disrupts the integrity of barrier but also increases the risk of colorectal carcinogenesis. Clinically, patients with UC often present with symptoms such as diarrhea, abdominal pain, bloody stool, and weight loss, which can progress to colorectal cancer, disability and impaired quality of life [3].

Although the exact etiology remains elusive, genetic predisposition, environmental factors, dysregulation of immune response, and gut microbiota dysbiosis are recognized as key contributors to UC pathogenesis [3]. A hallmark of UC is inflammatory response in intestinal mucosa, where proinflammatory cytokines- including interleukin-1beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) - play a significant role in disease progression [4,5]. Generation of cytokines and signal transduction initiated by cytokines were tightly regulated by intricate mechanisms. However, the pathophysiological dysregulation of gut immune response in UC resulted in imbalanced cytokines secretion, ultimately causing damage of gut mucosa [6]. Under physiological conditions, reactive oxygen species (ROS) are pivotal for cell signaling and tissue homeostasis [7]. However, in the context of inflammation in IBD, the imbalance of redox system resulted in excessive accumulation of ROS leading to cellular and molecular damages in the gut [8]. Oxidative stress is therefore considered as a key driver of the pathogenesis of UC and is closely associated with the progression of intestinal inflammation [8]. Additionally, gut microbiome formed a symbiotic relationship with the host, regulating intestinal immunity and gut function, and

its dysbiosis has been identified as a contributing factor to the pathogenesis of intestinal inflammation [9].

Currently, treatments primarily aim to alleviate symptoms of the patients, with clinical therapeutic strategies including 5-aminosalicylates, corticosteroids, thiopurines, pathway molecules inhibitors (e.g., TNF inhibitors, Janus kinase inhibitors), and probiotics [10]. Most existing therapies usually target a single mechanism, such as anti-inflammatory response or immunosuppressants [10]. Pulsatilla decoction (PD), a classical traditional Chinese medicine formula (CMF) recorded in the classical ancient Chinese book *Shang Han Treatise*, consists of four herbs: *Pulsatillae chinensis, Coptidis chinensis, Phellodendri Chinensis Cortex,* and *Fraxini Cortex*. It has long been used to treat diarrhea and IBD which alleviated UC in mouse model by reducing the tissue damage, inflammatory response and modifying the gut microbiota, and exhibits multi-target effect, low cost, and minimal side effects [11-13]. *Portulaca oleracea* L. (known as Ma Chi Xian in Chinese) is widely distributed globally, with a cold in nature and sour in taste in traditional Chinese medicine. It is traditionally used to cool the blood, stanch bleeding, antipyretic, and clear toxins [14]. Previous studies have shown that *Portulaca oleracea* L. traditional Chinese medicine compound or polysaccharides exerts therapeutic effects in UC [15,16].

Given these findings, the present study hypothesized that combining the classical PD with *Portulaca oleracea* L. (supplementary PD, SPD) may boost the therapeutic efficacy against UC. The aim of the present study is to investigate the effect of SPD on DSS-induced UC and explore its underlying mechanisms.

Material and Methods

Ethical Approval

The protocols of the trial involving animal experiments were reviewed and approved by Institutional Animal Use Committee of Henan Agricultural University (March 2nd 2022, HNND202203816), and the experiments were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* of the Ministry of Science and Technology. The number of the animals used in the experiment is rational and operations during the invasive experiment were performed under anesthesia to minimize the pain. Animals were euthanized at the end of the experiment to collect the samples.

Preparation of Supplemented Pulsatilla Decoction (SPD) Crude Extract

The supplemented pulsatilla decoction was composed of *Portulaca oleracea* L., *Pulsatillae chinensis, Coptis chinensis, Phellodendri Chinensis Cortex,* and *Fraxini Cortex*. Details of the herbs used in the present study were listed in *Table 1.* To prepare the crude extract of SPD, 2550 mL of distilled water was added to the mixed herbal materials of SPD and incubated for 30 min at room temperature. The mixture was then boiled for 30 min, and filtered through 4 layers of cotton gauze. The filtrate was collected and 2550 mL was added to the residue and boiled for an additional 30 min. The boiled mixture was filtered through 4 layers of cotton gauze and the filtrate was collected. The filtrates were combined, filtered through 8 layers of cotton gauze, and lyophilized with vacuum freeze dryer (Brocade Holding Group Limited, China) to obtain the SPD extract and was stored at -80°C.

Table 1. *Detailed information of herbs in SPD*

Chinese Name	Latin Name	Part (s) Used	Amount (g)
Ma Chi Xian	*Portulaca oleracea L.*	Stems and leaves	60
Bai Tou Weng	*Pulsatillae chinensis*	Roots	60
Huang Lian	*Coptis chinensis*	Roots and rhizomes	30
Huang Bai	*Phel lodendri Chinensis Cortex*	Epidermis	45
Qin Pi	*Fraxini Cortex*	Epidermis	60

LC-MS/MS Analysis

Supplemented pulsatilla decoction extract was diluted in double distilled water and subjected to LC-MS/MS analysis. The detection procedure was performed as previous description [17]. LC-MS/MS analysis was performed on an UHPLC system (Vanquish, Thermo Fisher Scientific) with a Waters UPLC BEH C18 column (1.7 µm 2.1*100 mm). An Q Exactive Focus mass spectrometer coupled with an Xcalibur software was employed to obtain the MS and MS/MS data based on the IDA acquisition mode. The original data of mass spectra were imported into XCMS software for processing such as retention time correction, peak identification, peak extraction, peak integration, and peak alignment. The peak information of compounds was searched through the in-house secondary mass spectrometry database provided by Shanghai BIOTREE biotech Co., Ltd.

Animals, Treatments, and Sample Collection

BABL/C male mice (6-8 weeks old, 20-24 g) were obtained from Beijing HFK Bioscience Co., LTD. Mice were housed individually in the individual ventilated cages (IVC) with free access to food and water. The housing temperature was maintained at 22°C with 50% humidity and a 12:12 light - dark cycle. The protocols of the trial were reviewed and approved by Institutional Animal Use Committee of Henan Agricultural University.

After the adaptive feeding for 7 days, 30 mice were randomly divided into 6 groups with 5 mice in each group and were treated as follows *(Fig. 2-a)*: control group (Ctrl); ulcerative colitis group (UC), where the ulcerative colitis mouse model was obtained by supplementing 3.5% dextran sulfate

sodium (DSS) in the drinking water for 7 days; sulfasalazine group (SF), where UC mice were treated with sulfasalazine (150 mg/kg) for 7 days; SPD treatment groups, where UC mice were treated either with high dose of SPD (SPDH, 17.29 g/kg), medium dose of SPD (SPDM, 8.75 g/kg) or low dose of SPD (SPDL, 4.37 g/kg) for 7 days. Sulfasalazine and different dose of SPD was given to the mice by intragastric gavage and all treatments were administered to each mouse within 1 minute. According to *Methodology of Traditional Chinese Medicine Pharmacological Research*, the adult dosage is about 1.9 g/kg per day, and the conversion coefficient of body weight for mice is 9.1. Thus, the normal gavage dosage of SPD is 9.1 x 1.9 = 17.29 g/kg, which was used as the high-dose SPD administration group. Serial dilution was performed to obtain the medium-dose (8.75 g/kg) and low-dose (4.37 g/kg) groups of the SPD. Weight of the mice and disease activity index (DAI) were evaluated according to the method descripted previously [18].

Mice were anesthetized with isoflurane and euthanized with cervical dislocation at the end of the 14th day on trial. Serum was collected by orbital venipuncture and the length of the colon was recoded. Colonic tissue about 1 cm in length was fixed in 4% formaldehyde and the remaining colon tissue was washed with 1 x PBS, fast frozen in liquid nitrogen, and then stored at -80°C. The content of the colon was sampled and stored in -80°C for microbiota analysis.

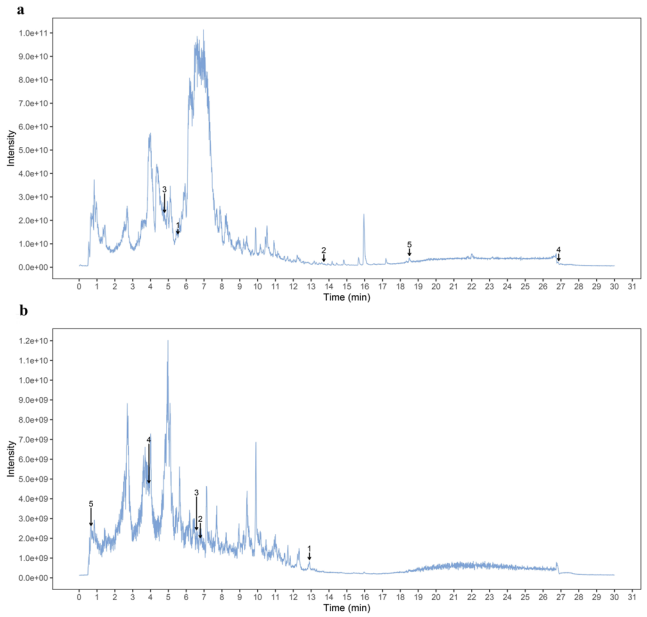

Fig 1. Representative components of SPD by UHPLC-QTOF-MS listed in *Table 1*. The total ion chromatograms (TIC) of SPD. **a-** The positive mode, **b-** the negative mode. Numbers indicate the representative compounds detected in SPD

H&E Staining Assay

Slides were prepared by the method described previously [19]. Briefly, the colon tissue fixed in formaldehyde were embedded in paraffin after dehydrated with different concentrations of ethanol and stained with H&E. Slides were visualized with a light microscope and captured using a high-resolution digital camera (Nikon Digital Sight DS-Fi1; Nikon Corporation, Minato-ku, Tokyo, Japan).

RNA Extraction, Synthesis of cDNA and Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from the colon tissue using RNA isolater Total RNA Extraction Reagent (Vazyme, Nanjing, Jiangsu, China) following the manufacturer's instruction. Concentrations of the isolated RNA were determined by NanoDrop2000 (Thermo Fisher Scientific Inc., Waltham, MA, United States). RNA was then reverse transcribed to cDNA using HiScript II Q RT SuperMix for qPCR kit (Vazyme, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Relative gene expression of *MUC2*, and *Occludin* were assessed by RT-qPCR assay using a ChamQ SYBR qPCR Master Mix Kit (Vazyme) according to the manufacturer's instruction. RT-qPCR was performed on a Quantitative Real-Time PCR instrument (Analytik jena, Jena, Germany) and the reaction conditions were as follow: 95°C for 30 s; 95°C 10 s→60°C 30 s, 40 cycles; 95°C 15 s→60°C 60 s→95°C 15 s. Relative gene expression was analyzed by normalizing to GAPDH (an internal control) which has been tested by a previous report [20]. Data (CT value) obtained from RT-qPCR was analyzed by the $2^{-\Delta\Delta Ct}$ calculation method as previously described [21]. Primers were commercially synthetized by Sangon Biotech (Shanghai) Co., Ltd., and the information of primers used in the present study was listed in *Table 2*.

Detection of MDA, SOD, CAT, and GSH

Enzymatic activity of superoxide dismutase (SOD) in the serum of the mice were measured by Total Superoxide Dismutase Assay Kit (Beyotime Biotechnology,

Table 2. *Information of primers used in the present study*

Gene Name	Gene ID	Primer sequence (5'-3')	Product Length (bp)
MUC2	NM_023566.4	Forward: AGGGCTCGGAACTCCAGAAA Reverse: CCAGGGAATCGGTAGACATCG	106
Occludin	NM_001360538.1	Forward: ACGGACCCTGACCACTATGA Reverse: TCAGCAGCAGCCATGTACTC	260
GAPDH	NM_001411843.1	Forward: TGGAGAAACCTGCCAAGTATGA Reverse: TGGAAGAATGGGAGTTGCTGT	135

Shanghai,China) following the instructions. Enzymatic activity of catalase (CAT) and reduced glutathione (GSH), and the level of malondialdehyde (MDA) in serum of mice were detected with the kit purchased from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China) according to the manufacturer's instructions. Detail information of the kits was presented in *Table 3*.

Enzyme-Linked Immunosorbent Assay (ELISA)

Protein levels of IL-1β, IL-6 and TNF-α in colonic tissue was measured by the kits obtained from Jiangsu Meimian Industrial Co., Ltd (Yancheng, China) according to the manufacturer's instructions. All experiments were replicated twice and data were calculated and normalized as the method described in the manufacturer's instruction. Detail information of the kits were listed in *Table 3*.

Gut Microbiota Analysis

Total genome DNA from samples was extracted using CTAB method. PCR amplification of the bacterial 16S rRNA genes 16S V4/16S V3/ 16S V3-V4 region was performed using the specific primers. Sequencing libraries were generated usingTruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit at 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2 1 0 0 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated at Novogene Biotech Co., Ltd. (Beijing, China).

After the sequencing data were spliced and quality-controlled, the optimized sequence was obtained. Based on the optimized sequence, operational taxonomic unit (OTU) clustering was performed by UPARSE ((Uparse v7.0. 1001, *http://drive5. com/uparse/)* at 97% similarity, and taxonomic information of OTU representative sequences was obtained for subsequent bioinformatics analysis. Relative abundance of top 10 species at phylum level and top 20 species at genus level was analyzed and depicted with perl-SVG software (NovoGene, Beijing, China). The linear discriminant analysis (LDA) effect size (LEfSe) method was used to find species with significant differences in abundance between groups. All analyses were performed on the NovoMagic cloud platform *(https://magic.novogene.com)*.

Statistics Analysis

Data except for body weight and DAI value were analyzed via one-way ANOVA with Dengken's post hoc test, using SPSS Statistics 26.0 for Windows (IBM Corp., Armonk, NY). Data of body weight and DAI value were analyzed with general liner model by SPSS Statistics 26.0. Normality distribution of variables were assessed using the Shapiro-Wilk test. Results were presented as mean ± standard error of the mean (SEM). The GraphPad Prism software 8.3.0 (Boston, Massachusetts USA) was used to display the data graphically. Significance was set at $P<0.05$.

Results

The Chemical Components of SPD

Representative chemical components were detected by UHPLC-QTOF-MS and the components were labeled with number in the total ion chromatograms (TIC) by the positive ESI+ *(Fig. 1-a)* or negative ESI− mode *(Fig. 1-b)*. Detail information of the representative chemical such as Hyoscyamine, Quinic acid and Salicylic acid. components labeled in the TIC were listed in *Table 4*.

Effect of SPD on Body Weight (BW) and DAI Value of the Mice

As shown in *Fig. 2-b,* BW of mice treated with DSS decreased dramatically from the 5th day compared with that of control group. The lowest BW of the mice in groups treated with DSS were observed on the 8th day. The weight gain was observed after the removal of DSS (on the 9th day on trial). Body weight of the mice in SF and SPDH group were higher relative to that in UC group from the 10th day till the end of the trail. Body weight of mice in SPDM group were higher relative to that of UC group from the 12th day till the 14th day.

As shown in *Fig. 2-c*, the highest DAI value was observed in mice of all groups treated with DSS on the 6th day of the trial. DAI value in mice of SF and SPDH groups was lower relative to the mice in UC groups from the 7th day till the end of the experiment. DAI value in mice of SPDL and SPDM group was lower from the 10th day till the end of the experiment.

Table 3. *Information of kits used in the present study*

Name of Kit	Catalog Number	Inter-assay CV	Intra-assay CV	Assay Range
Catalase (CAT)	A007-1-1	4.94	1.9	0.2-24.8 U/mL
Malondialdehyde (MDA)	A003-1-2	4.11	3.5	0-113.0 nmol/mL
Reduced glutathione (GSH)	A006-2-1	3.86	1.2	0.3-147.1 mg GSH/L
Mouse tumor necrosis α (TNF-α)	MM-0132M1	<12%	<10%	25 ng/L - 800 ng/L
Mouse interleukin 6 (IL-6)	MM-0163M1	<12%	<10%	3 pg/mL - 120 pg/mL
Mouse interleukin 1β (IL-1β)	MM-0040M1	<10%	<10%	3.75 pg/mL -120 pg/mL

Table 4. Representative bioactive compounds in SPD

NO.	Name	Formula	Class	RT (min)	Intensity
1	Hyoscyamine	C17H23NO3	Alkaloids	287.2	2514433851.0
2	Quinic acid	C7H12O6	Phenolic acids	39.9	1043744763.5
3	6,7-Dihydroxycoumarin	C9H6O4	Phenylpropanoids	234.9	505557176.2
4	Salicylic acid	C7H6O3	Phenols	394.7	367475974.3
5	Isokobusone	C14H22O2	Alcohol	774.2	221537844.1
6	7,8-dihydroxy-6-methoxychromen-2-one	C10H8O5	Phenylpropanoids	332.2	144166554.2
7	Betaine	C5H11NO2	Alkaloid	1612.8	71955250.4
8	Rosmarinic acid	C18H16O8	Phenylpropanoids	407.3	32940559.2
9	Neoandrographolide	C26H40O8	Terpenoids	1110.8	22078150.8
10	Cafestol	C20H28O3	Terpenoids	822.9	18558173.1

Effect of SPD on Colon Length and Histopathological Changes of the Colon

As shown in *Fig. 2-d,e,* colon length (CL) of the mice in UC group (6.90±0.089 cm) was significantly shorter than that of the mice in control group (8.34±0.081 cm, P<0.05). Colon length of mice in SPDM (7.54±0.068 cm, P <0.05) and SPDH (8.00±0.45 cm, P<0.05) group was longer compared with CL of mice in UC group (6.90±0.089 cm).

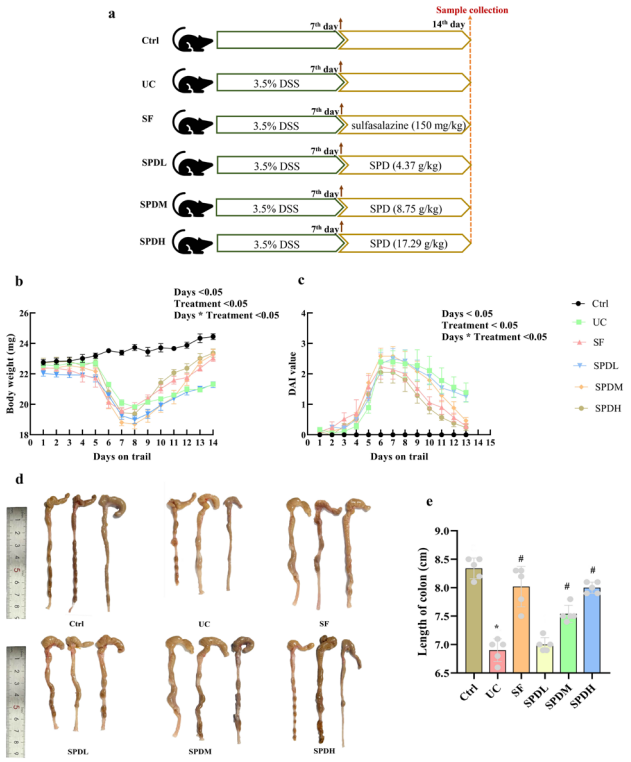

Fig 2. Effect of SPD on body weight, DAI index, and length of colon in DSS-induced UC mice. **a-** schematic diagram of the treatments, **b-** Body weight changes, P<0.05 indicates a significant difference, n=5, **c-** disease activity index (DAI), P<0.05 indicates a significant difference, n=5, **d-** Representative photos of colon, e- colon length in different groups. All data were expressed with mean ± SEM, * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

As depicted in *Fig. 3*, severe submucosal edema was observed in colon of mice in UC group compared with that in control group. Additionally, severe inflammatory cell infiltration in the mucosal layer and submucosa layer, intestinal gland atrophy and lysis was also observed in the colon of mice in UC group compared with that of control group. While the submucosal edema, inflammatory cell infiltration, and intestinal gland atrophy observed in the colon of the mice in UC group were dramatically decreased with the treatment of SF, SPDM and SPDH *(Fig. 3)*.

SPD Ameliorated UC by Inhibiting Proinflammatory Cytokine Expression and Enhancing Barrier Function

As shown in *Fig. 4-a,c*, protein concentration of IL-1β, IL-6 and TNF-α in colon tissue of the mice were significantly higher in UC group compared with that of Ctrl group (P<0.05). Protein concentration of IL-1β and IL-6 in colon tissue of the mice in SF, SPDL, SPDM, and SPDH groups were lower relative to

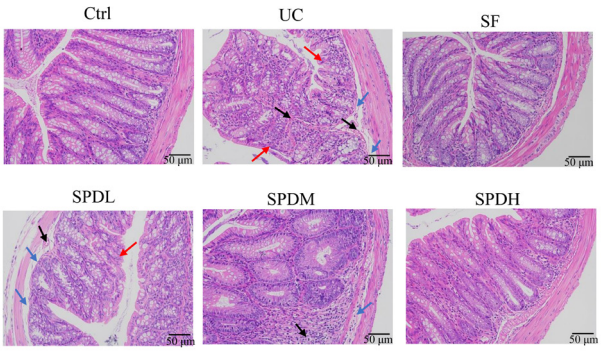

Fig 3. Representative sections from each group stained with H&E. Sections of different group were prepared and stained with H&E. The magnification was 200x and the scale bar was 50 μm. The red arrow indicates the atrophy and lysis of the intestinal glands. The black arrow represents inflammatory cell infiltration. The blue arrow represents tissue edema. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

that of UC group (P<0.05). Protein concentration of TNF-α in colon tissue of the mice in SF, SPDL and SPDH groups were lower compared with that of UC group (P<0.05).

Transcription of *mucin 2 (MUC2)* and *Occludin* was significantly lower in colon tissue of the mice in UC group compared with that of Ctrl group *(Fig. 4-d,e,* P<0.05). While transcription of MUC2 and Occludin was higher in colon tissue of the mice in SF, SPDM and SPDH groups compared with that of UC group *(Fig. 4-d,e,* P<0.05).

SPD Ameliorated the Oxidative Stress in Colon Induced by UC

Concentration of MDA in peripheral blood of mice in UC group was significantly higher compared with that of Ctrl group *(Fig. 5-a,* P<0.05). While the level of MDA decreased in peripheral blood of the mice in SF and SPDL, SPDM, and SPDH compared with that of UC group *(Fig. 5-a,* P<0.05). The enzymatic activity of GSH, SOD and CAT was lower in peripheral blood of mice in UC group compared with that of Ctrl group *(Fig. 5-b,d,* P<0.05). The enzymatic activity of GSH was higher in peripheral blood of the mice in SF, SPDM and SPDH groups compared with that in UC group *(Fig. 5-b,* P<0.05). The enzymatic activity of SOD was higher in peripheral blood of the mice in SF, SPDL, SPDM and SPDH groups relative to that of UC group *(Fig. 5-c,* P<0.05). The enzymatic activity of CAT in peripheral blood of the mice in SF and SPDH groups was higher compared with that of UC group (P<0.05).

SPD Ameliorated UC by Modulating the Microbiota in the Colon

As depicted in *Fig. 6-a,* the relative abundance of Proteobacteria was higher in the colon of the mice in UC group compared with that of the Ctrl group, while the abundance was decreased in response to SF and high dose of SPD treatment at phylum level *(Fig. 6-a)*. At genus level abundance of *Bacteroides*, *Parabacteroides* and *Prevotellaceae_UCG-001* was higher in the colon of the mice in UC group compared with that of the Ctrl group, but the abundance of these genus was lower in SF and SPDH group *(Fig. 6-b)*.

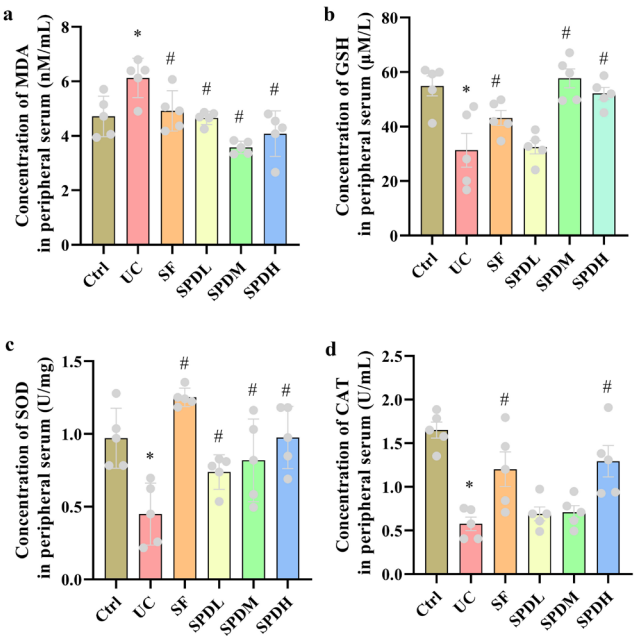

Fig 5. Effect of SPD on antioxidation index. Concentration of **(a)** MDA, **(b)** GSH, **(c)** SOD, and **(d)** CAT in peripheral blood of mice in different groups. All data were expressed with mean ± SEM. * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05, n=5. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

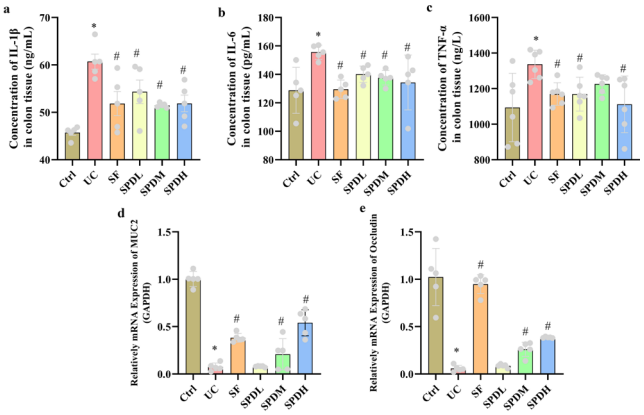

Fig 4. Effect of SPD on the level of proinflammatory cytokines and transcription of barrier proteins. Protein level of **(a)** IL-1β, **(b)** IL-6, **(c)** TNF-α and relative mRNA expression of **(d)** MUC2 and **(e)** Occludin in colon tissue of different groups. All data were expressed with mean ± SEM. * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05, n=5. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDL, low dose of SPD; SPDM, medium dose of SPD; SPDH, high dose of SPD

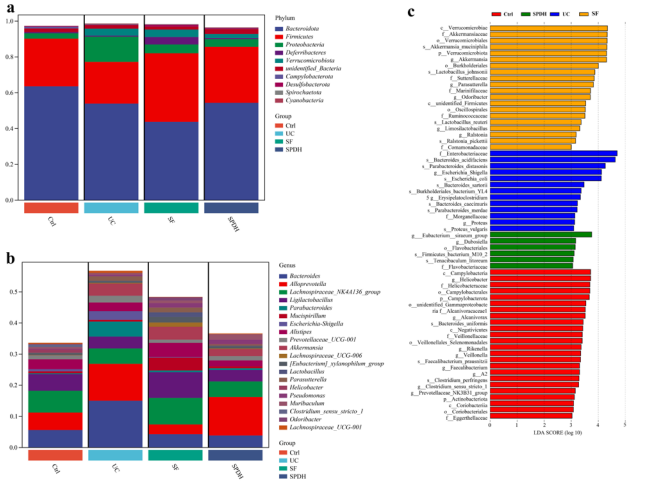

Fig 6. Effect of SPD on microbiota of mice with UC. **a**- Top 10 percent of community abundance of microbiota at phylum level, **b**- Top 20 percent of community abundance of microbiota at genus level, **c**- The LEfSe analysis of microbial abundance among groups, and LDA scores of bacterial taxa that were significantly enriched in each group (LDA score >3). Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDH, high dose of SPD

The representative species were further evaluated by LEfSe analysis. As presented in *Fig. 6-c,* genus *Helicobacter*, phylum Campylobacterota and other 22 species were significantly enriched in colon of the mice in Ctrl group. Genus *Escherichia_Shigella*, family *Enterobacteriaceae* and other 11 species were significantly enriched in colon of the mice in UC group. Phylum *Verrucomicrobiota,* genus *Akkermansia* and other 18 species were enriched in SF group and 6 species at genus level including: *Eubacterium__siraeum_group*, *Dubosiella*, *Flavobacteriales*, *Firmicutes_bacterium_M10_2*, *Tenacibaculum_litoreum* and *Flavobacteriaceae* were significantly enriched in colon of the mice in SPDH group.

Eight microbial species at genus level were found significantly modified in colon of the mice in Ctrl, UC, SF and SPDH groups *(Fig. 7-a)*. Furthermore, relative abundance of genus *Akkermansia* was significantly higher in in colon of the mice in UC and SF groups compared with that of Ctrl group *(Fig. 7-b,* P<0.05). While relative abundance of genus Akkermansia was lower in in colon of the mice in SPDH group compared with that of UC group *(Fig. 7-b,* P=0.2) and relative abundance of Ak*kermansia* in colon of the mice in SPDH group was similar compared with that of Ctrl group *(Fig. 7-b,* P>0.05).

Correlation Analysis Between Microbiota and Expression of Proinflammatory Cytokines, Antioxidant Enzymes and Tight-Junction Molecules

The results showed that the abundance of Bacteroidota was negatively associated with the level of IL-1β and positively associated with the level of CAT at phylum level *(Fig. 8-a)*. The level of IL-1β was positively associated

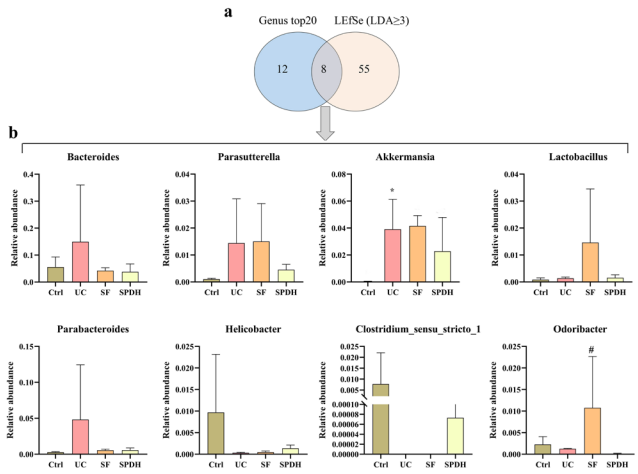

Fig 7. Differential gene screening and differential analysis. **a**- Venn Diagrams for LEfSe analysis and Random Forest Analysis, **b**- Relative abundance of differential microorganisms in different groups. All data were expressed with mean ± SEM. * indicates significant change vs Ctrl group, P<0.05; # indicates significant change vs UC group, P<0.05, P<0.05, n=4. Ctrl, control group; UC, ulcerative colitis group; SF, sulfasalazine group; SPDH, high dose of SPD

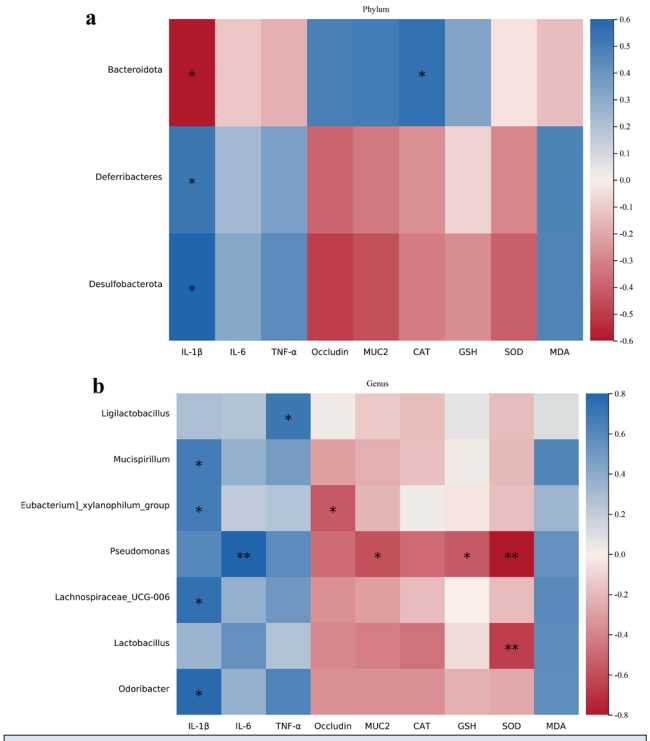

Fig 8. Spearman correlation analysis between intestinal microflora and inflammatory cytokines, barrier molecules and antioxidant enzymes. **a**- Phylum level, **b**- Genus level. Red color indicates negative correlation and bule color indicates positive correlation. * P<0.05, ** P<0.01, n=4

with abundance of Deferribacteres and Desulfobacterota at phylum level *(Fig. 8-a)*. The level of IL-1β was positively associated with abundance of *Odoribacter, Lachnospiraceae_UCG-006*, *Eubacterium]_xyanophilum_group* and *Mucispirillum* at genus level *(Fig. 8-b)*. The level of TNF-α was positively associated with the abundance of *Ligilactobacillus* at genus level *(Fig. 8-b)*. The abundance of *Pseudomonas* was positively associated with the level of IL-6, but was negatively associated with the level of *MUC2*, GSH and SOD at genus level *(Fig. 8-b)*. Relative mRNA level of *Occludin* was negatively associated with the abundance of *Eubacterium] _ xyanophilum_group* at genus level *(Fig. 8-b)*.

Discussion

Ulcerative colitis is a recurrent and relapsing disease, and its etiology is still not fully elucidated. Consequently, extensive studies have focus on developing novel therapeutic strategies for UC. Clinical manifestations, such as diarrhea, hematochezia, and weight loss are always observed in animals with UC [22]. In the present study, SF and SPD at different doses alleviated the lesions (increased DAI value and weight loss) induced by DSS. These findings confirm the therapeutic potential of SPD against UC. Subsequently, the present study investigated the underlying mechanisms of the therapeutic potential of SPD against UC.

Inflammation and immune dysregulation played key roles in the pathogenesis of UC, particularly involving cytokines and their regulatory pathways [5,23]. The present study shows that SPD treatment attenuated the UC-induced elevation of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. Elevated proinflammatory cytokines trigger immune cells activation, infiltration, and the initiation of inflammatory cascades [24]. Consistent with this, morphological analysis revealed reduced immune cell infiltration and tissue damage in SPD-treated groups, confirming that SPD mitigates intestinal inflammation by suppressing proinflammatory cytokine expression.

Intestinal barrier function of the gut is critical for the gut homeostasis, comprising physical barriers such as the mucus layer (composed of mucins secreted by goblet cells) and intercellular junctional complexes linking adjacent epithelial cells [25]. Mucin 2 is the major structural components of the intestinal mucus layer [26]. Occludin, a tetraspan integral membrane protein, contributes to the stability of tight junctions (TJs) [27]. The upregulation of *MUC2* and *Occludin* mRNA levels in SPD group suggested that SPD might ameliorated UC by enhancing the intestinal barrier function. However, further study is still needed to analyze the protein expression of MUC2 and occludin to validate these transcriptional changes which is the limitation of the present study.

Oxidative stress is well-recognized a pathogenic factor in UC and excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during chronic inflammation induces tissue damage [28]. Previous study shows that DSS treatment significantly reduces the enzymatic activity of CAT, GSH and SOD in mouse colon tissue [29], which is in line with results of the present study. The body's antioxidant defense system comprises three layers: small antioxidants molecular (e.g., uric acid, glutathione), antioxidant enzymes and damage-repairing enzymes [30]. Antioxidant enzymes exert protective effects by detoxifying ROS/RNS into less reactive metabolites. Superoxide dismutases and CAT are key antioxidant enzymes responsible for scavenging superoxide (O^{2-}) and hydrogen peroxide (H_2O_2), respectively [31,32]. So, down regulation of SOD, CAT and GSH activity leads to ROS accumulation, inducing damage of biologically important molecules (e.g., lipids) or cells. In the present study, SPD inhibited DSS-induced elevation of MDA and reverse the reduction in the activity of CAT, GSH and SOD, indicating that SPD ameliorated UC by enhancing antioxidant enzymes activity and mitigating oxidative stress. Furthermore, mass spectrometry analysis of SPD identified major bioactive components -including quinic acid, 7,8-dihydroxy-6-methoxychromen-2-one (fraxetin) and salicylic acid- all of which had been reported to possess anti-inflammatory and antioxidant properties [33,34]. These effective components in SPD could contribute to the therapeutic efficacy of SPD against UC.

Dysbiosis of gut microbiota is a well-established key factor in the pathogenesis of UC [35,36]. Previous study has shown that increased abundance of phylum Proteobacteria DSS-induce UC mice [22], which was also observed in the present study. Notably, high-dose SPD treatment reduced the DSS-induced overabundance of phylum Proteobacteria, which is closely associated with inflammatory diseases [37]. Genus *Bacteroides* is a commensal gut bacterium, while overabundance of *Bacteroides* was associated with increased degradation of intestinal mucus and compromised barrier function [38]. Genus *Parabacteroides*, a group of gram-negative anaerobic bacteria that colonizes the gastrointestinal tract of various species, has also been implicated in the pathogenesis of IBD [39]. In the present study, high-dose SPD reduced the DSS-induced elevation of genus *Parabacteroides* and *Bacteroides* abundances, which may contribute to its protective effects on intestinal barrier function and UC alleviation. Additionally, DSS-induced mice exhibited an increase in relative abundance of genus *Akkermansia*, genus *Akkermansia* could born inside the GI tract and possessed the ability to degrade mucin [40], while high-dose SPD reversed this increase. Collectively, these results demonstrate that SPD alleviated UC by modulating gut microbiota dysbiosis.

Correlation analysis revealed that pathogenic bacteria species was positively correlated with proinflammatory cytokines level and negatively correlated with the antioxidant enzymes activity and key intestine barrier molecules *(MUC2, occludin)*. These findings suggest tight crosstalk between gut microbiota alterations, inflammatory responses, antioxidant capacity, and intestinal barrier function in UC. However, the underlying detailed mechanisms required further investigation. Oxidative stress is reported to interrelated with inflammation. Decreased antioxidant enzyme activity results in the accumulation of ROS which promotes the proinflammatory response by modulating NF-κB signaling [41,42]. A limitation of the present study is the absence of treatment group of traditional PD group alone and *Portulaca oleracea L* alone, which would have enabled evaluation of whether their combination could synergistically enhance the therapeutic efficacy against UC.

In conclusion, SPD meliorated the DSS-induced UC by suppressing the expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and enhancing the activities of antioxidant enzymes (SOD, GSH and CAT), and the expression of key intestinal barrier molecules *(MUC2* and *Occludin)* and modulating gut microbiota dysbiosis.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (L. F.).

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Ethical Approval: The protocols of the trial involving animal experiments were reviewed and approved by Institutional Animal Use Committee of Henan Agricultural University (March 2nd 2022, HNND202203816).

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

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Author Contributions: Conceptualization/Resources/Supervision/Project administration/Funding acquisition, HD, FL and HJD; Formal analysis, HD; Investigation, ZC, HW, JG, GC, LL, KZ, SZ, PS; Methodology, HD and ZC; Writing - original draft, HD, ZC and FL; Writing - review & editing, FL and HJD.

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Research Article

Prevalence of *Anaplasma* and *Ehrlichia* Infection with Molecular Characterization of *Anaplasma* Species in Cattle from Northeastern Anatolia, Türkiye

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Abstract

This study aimed to molecularly identify and characterize *Anaplasma* species and detect *Ehrlichia* species in cattle from Kars, Ardahan, and Iğdır provinces. Blood samples from 1000 clinically healthy cattle were analyzed using PCR and Reverse Line Blotting (RLB) techniques. The prevalence of *Anaplasma* and *Ehrlichia* spp. and their association with age, sex, and breed were evaluated using the "prevalence" package (version 0.2.0) and Pearson's chi-square test. The results revealed that only *Anaplasma* species (36.6%) were detected, whereas *Ehrlichia* spp. were not found in any of the samples. The species distribution was as follows: *A. marginale* (17.9%), *A. phagocytophilum* (14.0%), and *A. bovis* (4.2%). Mixed infections were observed in 1.37% of the cases. *Anaplasma marginale* was most frequently detected in Kars (23.60%) and Iğdır (10.66%), while *A. phagocytophilum* was most prevalent in Ardahan (23.28%). Sequence analysis of *A. marginale*, *A. phagocytophilum*, and *A. bovis* isolates showed 100% identity with previously published sequences in GenBank. In conclusion, the detection of *A. marginale*, *A. phagocytophilum*, and *A. bovis* in cattle demonstrates that the ecological and epidemiological conditions in Northeastern Anatolia are favorable for the circulation of these pathogens. The high prevalence of *A. marginale* underscores its potential impact on regional livestock health. *Anaplasma phagocytophilum* may represent a threat not only to cattle but also to public health in the region.

Keywords: *Anaplasma*, Cattle, *Ehrlichia*, Molecular detection, Prevalence

Introduction

Ticks are considered the second most important arthropod vectors after mosquitoes, surpassing other hematophagous arthropods in their ability to transmit a wide range of pathogens. They are known to host at least 83 viral, 31 bacterial, and 32 protozoan species [1]. *Anaplasma* and *Ehrlichia* species are obligate intracellular, tick-borne rickettsial microorganisms belonging to the family Anaplasmataceae [2]. These pathogens pose significant health threats to domestic animals, wildlife, and human populations worldwide [3-5]. These obligate intracellular bacteria are transmitted by ticks and cause a variety of clinical disorders in ruminants, canines, and humans, often resulting in significant economic losses and public health concerns [5,6]. Among *Anaplasma* species, *A. phagocytophilum* and *A. marginale* are the primary pathogens affecting cattle, leading to febrile illnesses characterized by hemolytic anemia, decreased milk yield, respiratory signs, and reproductive disorders. In contrast, *A. bovis* and *A. centrale* are considered less pathogenic and typically result in subclinical infections [7]. Bovine ehrlichiosis is caused by several species of *Ehrlichia*, primarily transmitted by various species of hard ticks. The common species affecting large ruminants include *Ehrlichia bovis*, *E. ondiri*, *E. chaffeensis*, and *E. ruminantium* [8,9]. Clinical signs include irregular fever, ear drooping, turning movements, and lymphadenitis. Some studies have reported high mortality occurring within a few hours in the peracute stage and within 36–48 hours in the acute stage, although the disease often remains subclinical [10].

Although there is a serological study reporting a 52.1% seroprevalence of *Anaplasma marginale* in cattle in the Kars region [11], no molecular epidemiological data are

currently available for the provinces of Kars, Ardahan, and Iğdır. In contrast, several molecular studies on *Anaplasma* species in cattle have been conducted in other regions of Türkiye [7,12-15]. Given the growing importance of molecular identification of pathogens that infect veterinary animals, as emphasized in recent studies [16], such investigations are essential for understanding epidemiology and improving disease control strategies. The combination of favorable climatic conditions, geographic features, and widespread pasture-based livestock production in these provinces creates a suitable ecological niche for tick proliferation, suggesting a high potential for the transmission of tick-borne pathogens.

Therefore, the aim of this study was to molecularly identify the presence of *Anaplasma* and *Ehrlichia* species in cattle across the provinces of Kars, Ardahan, and Iğdır, to genetically characterize the positive samples obtained, and to update regional prevalence data. This study also acknowledges its regional scope, which may limit broader generalization of the findings, yet provides essential baseline data for future large-scale epidemiological investigations.

Material and Methods

Ethical Statement

All procedures involving animals were approved by the Kafkas University Local Ethics Committee on Animal Experiments (Decision no: KAÜ-HADYEK 2022/099), and sampling was conducted with permission from the Provincial Directorate of Agriculture and Forestry.

Study Region and Sample Collection

This study was conducted in the northeastern Anatolia region of Türkiye, specifically in the provinces of Kars, Ardahan, and Iğdır. Between 2022 and 2023, a total of 1.000 blood samples were collected from cattle raised on randomly selected farms engaged in bovine husbandry across various districts of each province *(Fig. 1)*. Epidemiological data, including the animals' age, sex, breed, and geographic location, were also recorded.

Fig 1. The geographic distribution of blood samples collected from 64 villages across the provinces of Kars, Ardahan, and Iğdır. Each point represents a sampling location in a specific village (generated using QGIS)

Inclusion and Exclusion Criteria

Clinically healthy cattle without recent antibiotic treatment were included in the study. Samples with insufficient epidemiological data or poor sample quality were excluded.

Blood Sample Collection

A total of 1.000 blood samples were collected from clinically healthy or asymptomatic cattle. Approximately 5 mL of blood was drawn aseptically from either the jugular vein or coccygeal vein using standard techniques and transferred into EDTA-containing tubes. The samples were transported to the laboratory at 4°C and stored at -20°C until DNA extraction was performed.

DNA Extraction, PCR Amplification, and Reverse Line Blotting (RLB) for the Detection of *Anaplasma* and *Ehrlichia* Species

Genomic DNA was extracted from blood samples using a commercial kit (EcoPURE Blood Genomic DNA Kit, Cat. No: E1075-50x, Türkiye) according to the manufacturer's instructions. The extracted DNA samples were stored at −20°C until molecular analyses were performed.

Reverse Line Blotting (RLB) is a two-step technique. In the first step, target gene amplification is performed by PCR, and in the second step, species identification is achieved through hybridization with species-specific oligonucleotide probes.

The variable V1 region (492-498 bp) of the 16S rRNA gene from *Anaplasma* and *Ehrlichia* species was amplified in the first PCR step using primers 16S8FE and BGA1B [17].

To reduce non-specific amplification, a touchdown PCR protocol was employed. The program began with an initial denaturation at 94°C for 5 min, followed by cycles consisting of denaturation at 94°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 30 sec. These steps were repeated in two cycles each at progressively decreasing annealing temperatures of 60, 58, 56, 54, 52, and 50°C. Subsequently, 40 additional cycles were performed with denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 5 min.

A positive control DNA and distilled water were used as negative controls in the PCR reactions. Each PCR reaction was prepared in a final volume of 25 µL, containing 2 µL of DNA template, 12.5 µL of 2X PCR Master Mix (EcoTaq), 1 µL of each primer (10 pmol), and 8.5 µL of nuclease-free water. Thermal cycling was performed under appropriate conditions as described above.

Five microliters of the PCR product were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized under UV transillumination. The remaining PCR amplicons were stored at -20°C until hybridization for Reverse Line Blotting (RLB). Probes containing an N-terminal N-(trifluoracetamidohexyl-cyanoethyl, N,N-diisopropylphosphoramidite [TFA])-C6 amino linker were synthesized by Macrogen (Korea) and used at concentrations ranging from 200 to 900 pmol per 150 µL hybridization mixture *(Table 1)*. The preparation of the Biodyne C membrane, hybridization procedures, and post-hybridization washing steps were performed as previously described [18]. Chemiluminescent signal detection was carried out using the ChemiDoc™ MP Imaging System (Bio-Rad, USA), and the presence of positive signals was interpreted based on the appearance of black dots at specific probe positions.

Sequencing and Phylogenetic Analysis of Positive Samples

Selected PCR- and RLB-positive samples were subjected to nucleotide sequencing of the 16S SSU rRNA gene [22,23] and the 16S rRNA major surface protein-1b (msp1b) gene [24] for species confirmation and genetic characterization. Sanger sequencing of the purified amplicons was performed by Medsantek Inc. (Istanbul, Türkiye). The quality scores of the obtained chromatograms were assessed using Geneious® version 9.1.8 software.

The forward and reverse nucleotide sequences obtained from each primer were aligned with reference sequences available in GenBank using the MAFFT algorithm (version 7.526), and consensus sequences were generated. The consensus sequences obtained in this study were submitted to GenBank, and corresponding accession numbers were obtained. Additionally, the consensus sequences of the positive samples were compared with similar pathogen sequences deposited in GenBank using the BLAST (Basic Local Alignment Search Tool) algorithm to assess nucleotide similarity.

Anaplasma 16S rRNA gene sequences obtained from the NCBI database were aligned using MAFFT version 7.526, and the best-fitting substitution models were selected for each aligned dataset using JModelTest version 2.1.10, based on the Akaike Information Criterion (AIC). The TPM1uf+G model was selected for Anaplasma bovis while the GTR+G model was identified as the best fit for A. marginale and A. phagocytophilum. Phylogenetic trees were reconstructed using the Maximum Likelihood (ML) method implemented in IQ-TREE multicore version 2.4.0, with 1000 bootstrap replicates to assess branch support. The resulting trees were rooted using appropriate outgroup sequences and visualized using FigTree version 1.4.4.

Statistical Analysis

All statistical analyses in this study were performed using the R programming language [25]. For data processing and analysis, the epiR package (version 2.0.62) was employed [26], which facilitates the calculation of core epidemiological metrics such as sensitivity, specificity, and prevalence. The Wilson method [27] was used to calculate confidence intervals for prevalence and diagnostic accuracy estimates. This method was preferred over the traditional Wald method, as the latter tends to be unreliable for small sample sizes or extreme proportion values. The Wilson method, derived from the binomial distribution, provides more accurate and asymmetric interval estimates. Associations between *Anaplasma/Ehrlichia* positivity and variables such as province, age group, breed, and sex were assessed using the Pearson chi-square (χ^2) test. This significance refers specifically to the comparison of prevalence rates among the three provinces (Kars, Ardahan, and Iğdır). A P-value less than 0.05 was considered statistically significant.

Results

This study was conducted by collecting blood samples from a total of 1.000 cattle across 64 villages in the provinces of Kars, Ardahan, and Iğdır, located in the Northeastern Anatolia Region of Türkiye. The sampling distribution was as follows: 483 cattle from 25 villages in Kars, 292 cattle from 25 villages in Ardahan, and 225 cattle from 14 villages in Iğdır.

Molecular Detection Results

PCR analysis using the primers 16S8FE and BGA1B identified 42 positive samples out of 1000 cattle,

Table 1. *Oligonucleotide Probes Used in Reverse Line Blotting (RLB)*

Probe	Sequences (5'-3')	Reference
Catchall (*Anaplasma* spp.+*Ehrlichia* spp.)	Amino-GGG GGA AAG ATT TAT CGC TA	[19]
Anaplasma marginale	Amino-GAC CGT ATA CGC AGC TTG	[19]
Anaplasma centrale	Amino-TCG AAC GGA CCA TAC GC	[19]
Anaplasma bovis	Amino-GTA GCT TGC TAT GRG AAC A	[20]
Anaplasma (E.) phagocytophylum	Amino-TTG CTA TRR AGA ATA RTT AGT GG	[21]
E. ruminantium	Amino-AGT ATC TGT TAG TGG CAG	[19]
E. chaffeensis	Amino-CC TTT TGG TTA TAA ATA ATT GTT	[17]

corresponding to a positivity rate of 4.2%. Following Reverse Line Blotting (RLB) analysis, the overall detection rate increased to 36.6%.

Among the identified species, *Anaplasma marginale* was detected in 17.9% of the samples, followed by *A. phagocytophilum* in 14.0%, and *A. bovis* in 4.2%. Additionally, mixed infections involving two or more species were identified in 1.37% of the samples. No *Ehrlichia* spp. were detected in any of the analyzed samples.

Regional Distribution of *Anaplasma* Species

The prevalence of *Anaplasma* species detected in this study exhibited regional variation. The highest prevalence was observed in Kars province (19.5%), followed by Ardahan (12.6%) and Iğdır (4.5%). In addition, the detection of mixed infections in 1.37% of the samples suggests the co-circulation of multiple *Anaplasma* species within the same geographic area *(Table 2)*.

Based on the data obtained in this study, no statistically significant association was found between *Anaplasma* species and the variables of age, breed, or sex overall (P>0.05). However, when stratified by province, a significant association was detected between age and *Anaplasma* species in Kars, and between sex and *Anaplasma* species in Iğdır (P<0.05). In contrast, breed was not significantly associated with *Anaplasma* positivity in any of the provinces *(Table 3, Table 4, Table 5)*.

DNA Sequencing and Phylogenetic Analysis

To achieve molecular characterization, genotyping, and phylogenetic analysis of the *Anaplasma* species identified in this study, DNA sequencing was performed on selected PCR-positive samples. The *Anaplasma marginale, A. phagocytophilum,* and *A. bovis* species identified by Reverse Line Blotting (RLB) were subjected to nucleotide sequencing of the 16S SSU rRNA variable region and the major surface protein 1b (msp1b) gene. The resulting consensus sequences were submitted to GenBank and assigned the following accession numbers: *A. marginale* - PV569600, *A. bovis* - PV569601, *A. phagocytophilum* - PV569602.

Phylogenetic trees were constructed using the 16S SSU rRNA and msp1b gene sequences of these species: *Fig. 2: A. marginale, Fig. 3: A. phagocytophilum, Fig. 4: A. bovis.*

The Maximum Likelihood (ML) method was applied using IQ-TREE (multicore version 2.4.0), with 1000 bootstrap replicates to assess node reliability. Tree visualization was performed with FigTree version 1.4.4.

Discussion

In this study, molecular analyses were performed on blood samples collected from a total of 1,000 cattle in the provinces of Kars, Ardahan, Iğdır, and DNA belonging to *Anaplasma marginale*, *Anaplasma bovis*, and *Anaplasma phagocytophilum* was detected. No *Ehrlichia* species were identified in any of the samples. The application of the Reverse Line Blotting (RLB) technique, combined with

Table 2. *Distribution of Anaplasma species detected in cattle from Kars, Ardahan, and Iğdır provinces*

Province	*Anaplasma* Species	Positive Samples	N	%Prev (95% CI)	P	χ^2	Mean
Kars	*A.bovis*	22	483	4.55 (3.02-6.79)	0.0000000241	35.08	P<0.05
	A. marginale	114		23.60 (20.03-27.58)			
	A. phagocytophlum	55		11.38 (8.85-14.52)			
	A. bovis/A. marginale	3		0.62 (0.21-1.81)			
	A. bovis/A. phagocytophilum	1		0.20 (0.01-1.16)			
Ardahan	*A. bovis*	16	292	5.47 (3.40-8.71)			
	A. marginale	41		14.04 (10.52-18.49)			
	A. phagocytophlum	68		23.28 (18.80-28.46)			
	A. bovis/A.marginale	1		0.34 (0.01-1.91)			
	A.bovis/A. phagocytophilum	0		0 (0-1.29)			
Iğdır	*A. bovis*	4	225	1.77 (0.69-4.48)			
	A. marginale	24		10.66 (7.27-15.38)			
	A. phagocytophlum	17		7.55 (4.77-11.76)			
	A. bovis/A. marginale	0		0 (0-1.67)			
	A. bovis/A. phagocytophilum	0		0 (0-1.67)			

N: *Number of sample,* ***CI:*** *Confidence intervals,* ***Prev:*** *Prevalence, Statistical significance was defined as P<0.05*
The value "0.0000000241" represents a prevalence proportion, not a p-value

Table 3. *Distribution of Anaplasma species by sex in cattle from Kars, Ardahan, and Iğdır provinces*

Province	Species	Sex						P	χ^2	Mean
		Female			Male					
		Positive Samples	N	%Prev (95% CI)	Positive Samples	N	%Prev (95% CI)			
Kars	*A. bovis*	22	462	4.76 (3.16-7.10)	0	21	0 (0-15.46)	0.070	3.27	P>0.05
	A. marginale	112		24.24 (20.55-28.35)	2		9.52 (2.65-28.91			
	A. phagocytophlum	53		11.47 (8.87-14.70)	2		9.52 (2.65-28.91			
	A. bovis/A. marginale	3		0.64 (0.22-1.89)	0		0 (0-15.46)			
	A. bovis/A. phagocytophilum	1		0.21 (0.01-1.21)	0		0 (0-15.46)			
Ardahan	*A. bovis*	15	277	5.41 (3.30-8.74)	1	15	6.66 (0.34-29.81)	0.603	0.27	P>0.05
	A. marginale	38		13.71 (10.16-18.26)	3		20.00 (7.04-45.18)			
	A. phagocytophlum	67		24.18 (19.52-29.56)	1		6.66 (0.34-29.81)			
	A. bovis/A. marginale	1		0.31 (0.01-2.01)	0		0 (0-20.38)			
	A. bovis/A. phagocytophilum	0		0 (0-1.36)	0		0 (0-20.38)			
Iğdır	*A. bovis*	4	180	2.22 (0.86-5.57)	0	45	0 (0-7.86)	0.007	7.34	P<0.05
	A. marginale	22		12.22 (8.21-17.81)	2		4.44 (1.22-14.82)			
	A. phagocytophlum	17		9.44 (5.98-14.60)	0		0 (0-7.86)			
	A. bovis/A.marginale	0		0 (0-2.08)	0		0 (0-7.86)			
	A. bovis/A. phagocytophilum	0		0 (0-2.08)	0		0 (0-7.86)			

N: *Number of sample,* ***CI:*** *Confidence intervals,* ***Prev:*** *Prevalence, Statistical significance was defined as P<0.05*
The values 0.070, 0.603, and 0.007 correspond to prevalence proportions rather than p-values.

Table 4. *Distribution of Anaplasma species by age group in cattle from Kars, Ardahan, and Iğdır*

Province	Species	Age									P	χ^2	Mean
		0-12 Months			12-24 Months			24+ Months					
		Positive Samples	N	%Prev (95% CI)	Positive Samples	N	%Prev (95% CI)	Positive Samples	N	%Prev (95% CI)			
Kars	*A. bovis*	0	0	0 (0-0)	0	51	0 (0-7.00)	22	432	5.09 (3.38-7.58)	0.00002	18.08	P<0.05
	A. marginale	0		0 (0-0)	4		7.84 (3.09-18.49)	110		25.46 (21.58-29.77)			
	A. phagocytophlum	0		0 (0-0)	2		3.92 (1.08-13.21)	53		12.26 (9.50-15.69)			
	A. bovis/A. marginale	0		0 (0-0)	0		0 (0-7.00)	3		0.69 (0.23-2.02)			
	A. bovis/A. phagocytophilum	0		0 (0-0)	0		0 (0-7.00)	1		0.23 (0.01-1.29)			
Ardahan	*A. bovis*	0	2	0 (0-65.76)	1	41	2.43 (0.12-12.59)	15	249	6.02 (3.68-9.70)	0.152	3.76	P>0.05
	A. marginale	0		0 (0-65.76)	5		12.19 (5.32-25.54)	36		14.45 (10.62-19.36)			
	A. phagocytophlum	1		50.00 (2.56-97.43)	6		14,63 (6.88-28.44)	61		24.49 (19.57-30.20)			

Table 4. *Continue*

Province	Species	Age									P	χ^2	Mean
		0-12 Months			12-24 Months			24+ Months					
		Positive Samples	N	%Prev (95% CI)	Positive Samples	N	%Prev (95% CI)	Positive Samples	N	%Prev (95% CI)			
Ardahan	*A. bovis/A. marginale*	0	2	0 (0-65.76)	0	41	0 (0-8.56)	1	249	0.40 (0.02-2.23)	0.152	3.76	P>0.05
	A. bovis/A. phagocytophilum	0		0 (0-65.76)	0		0 (0-8.56)	0		0 (0-1.51)			
Iğdır	*A. bovis*	0	18	0 (0-17.58)	1	51	1.96 (0.10-10.30)	3	156	1.92 (0.65-5.50)	0.145	3.86	P>0.05
	A. marginale	0		0 (0-17.58)	4		7.84 (3.09-18.49)	20		12.82 (8.45-18.97)			
	A. phagocytophlum	1		5.55 (0.28-25.75)	3		5.88 (2.02-15.92)	13		8.33 (4.93-13.73)			
	A. bovis/A. marginale	0		0 (0-17.58)	0		0 (0-7.00)	0		0 (0-2.40)			
	A. bovis/A. phagocytophilum	0		0 (0-17.58)	0		0 (0-7.00)	0		0 (0-2.40)			

N: *Number of sample,* ***CI:*** *Confidence intervals,* ***Prev:*** *Prevalence, Statistical significance was defined as P<0.05*
The values 0.00002, 0.152, and 0.145 correspond to prevalence proportions rather than p-values.

Table 5. *Distribution of Anaplasma species by cattle breed in Kars, Ardahan, and Iğdır provinces*

Province	Species	Breed						P	χ^2	Mean
		Dual-Purpose			Dairy					
		Positive Samples	N	%Prev (95% CI)	Positive Samples	N	%Prev (95% CI)			
Kars	*A. bovis*	20	463	4.31 (2.81-6.57)	2	20	10.00 (2.78-30.10)	0.507	0.44	P>0.05
	A. marginale	111		23.97 (20.30-28.06)	3		15.00 (5.23-36.04)			
	A. phagocytophlum	51		11.01 (8.47-14.19)	4		20.00 (8.06-41.60)			
	A. bovis/A. marginale	2		0.43 (0.11-1.56)	1		5.00 (0.25-23.61)			
	A. bovis/A. phagocytophilum	1		0.21 (0.01-1.21)	0		0 (0-16.11)			
Ardahan	*A. bovis*	16	289	5.53 (3.43-8.80)	0	3	0 (0-56.14)	0.810	0.058	P>0.05
	A. marginale	40		13.84 (10.33-18.29)	1		33.33 (1.70-79.23)			
	A. phagocytophlum	67		23.18 (18.68-28.38)	1		33.33 (1.70-79.23)			
	A. bovis/A. marginale	1		0.34 (0.01-1.93)	0		0 (0-56.14)			
	A. bovis/A. phagocytophilum	0		0 (0-1.31)	0		0 (0-56.14)			
Igdır	*A. bovis*	4	198	2.02 (0.78-5.07)	0	27	0 (0-12.45)	1.000	0.00	P>0.05
	A. marginale	20		10.10 (6.63-15.08)	4		14.81 (5.91-32.47)			
	A. phagocytophlum	16		8.08 (5.03-12.72)	1		3.70 (0.18-18.28)			
	A. bovis/A. marginale	0		0 (0-1.90)	0		0 (0-12.45)			
	A. bovis/A. phagocytophilum	0		0 (0-1.90)	0		0 (0-12.45)			

N: *Number of sample,* ***CI:*** *Confidence intervals,* ***Prev:*** *Prevalence, Statistical significance was defined as P<0.05*
The values 0.507, 0.810, and 1.000 correspond to prevalence proportions rather than p-values.

sequencing, enabled more sensitive detection and accurate species differentiation compared to conventional PCR.

In recent years, numerous studies on vector-borne diseases conducted worldwide have demonstrated that the prevalence of such infections varies considerably across geographical regions. Molecular investigations targeting

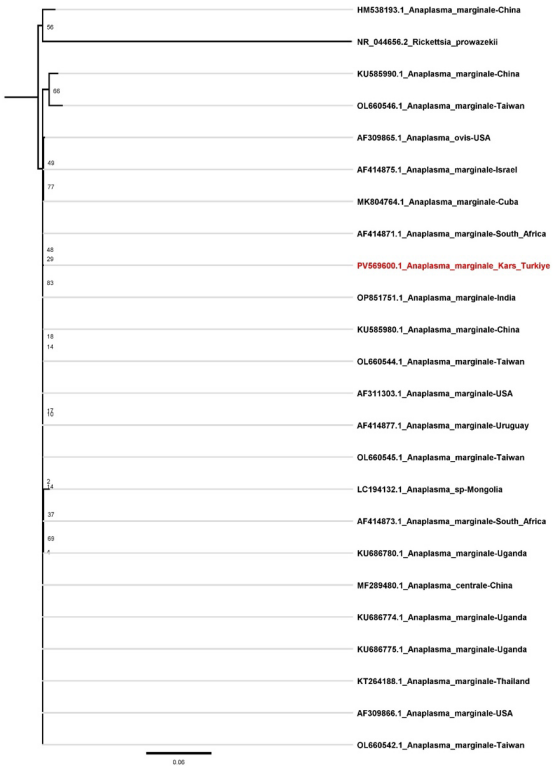

Fig 2. Maximum Likelihood phylogenetic tree of *Anaplasma marginale* constructed using IQ-TREE software based on the nucleotide sequences of the 16S rRNA major surface protein-1b (msp1b) gene. The tree is based on a multiple sequence alignment of partial msp1b gene sequences (265 bp) of *A. marginale* (GenBank accession no: PV569600)

Fig 3. Maximum Likelihood phylogenetic tree of *Anaplasma phagocytophilum* constructed using IQ-TREE software based on the nucleotide sequences of the 16S SSU rRNA gene. The tree is derived from a multiple sequence alignment of partial 16S SSU rRNA gene sequences (641 bp) of *A. phagocytophilum* (GenBank accession no: PV569602)

Fig 4. Maximum Likelihood phylogenetic tree of *Anaplasma bovis* constructed using IQ-TREE software based on the nucleotide sequences of the 16S SSU rRNA gene. The tree is based on a multiple sequence alignment of partial 16S SSU rRNA gene sequences (345 bp) of *A. bovis* (GenBank accession no: PV569601)

Anaplasma species have revealed prevalence rates ranging from 1.7% in Kyrgyzstan [28], 3.2% in China [29], and 20.8% in Thailand [30], to as high as 36% in Ecuador [31] and 87.3% in Colombia [32]. In Türkiye, reported prevalence rates range between 0% and 30.8% [33].

The growing importance of molecular identification of pathogens in veterinary medicine has been emphasized in previous studies [16], and our results support the need for continued molecular surveillance to better understand epidemiological patterns and improve disease control strategies. In the present study, the overall prevalence of *Anaplasma* species was found to be 36.6%, which is relatively high compared to several previous reports. This variation may be attributed to differences in ecological and geographical conditions, vector distribution, host susceptibility, and importantly, the sensitivity of the molecular detection techniques employed.

Geographical studies on bovine anaplasmosis in Türkiye have demonstrated that the distribution and dynamics of *Anaplasma* species vary significantly across regions [7,15,33-35]. In the Black Sea Region, *A. phagocytophilum* has been reported with a prevalence of 30.8%, while *A. marginale* and *A. bovis* were detected at rates ranging from 2.8% to 18.8% and 0.7% to 1%, respectively [34,35]. A study from the Eastern Anatolia Region, specifically in Malatya, found

A. marginale at a prevalence of 32.5%, along with the detection of other *Anaplasma* species [7]. In the Aegean Region, *A. phagocytophilum* was detected at a prevalence of 5% [15]. Furthermore, a large-scale study covering 16 provinces of Türkiye reported prevalence rates of *A. marginale* (10.5%), *A. phagocytophilum* (13.8%), *A. bovis* (0.5%), and other *Anaplasma* species (2.9%) in the Central and Southeastern Anatolia regions [25]. In the current study, *A. marginale* showed the highest prevalence in Kars (23.6%) and Iğdır (10.66%), while *A. phagocytophilum* was most frequently detected in Ardahan (23.28%). These differences may be attributed to local ecological conditions, the density and species composition of tick populations, livestock management practices, and other environmental factors. The unique environmental characteristics of each province likely influence tick activity and pathogen transmission, thereby contributing to regional variation in prevalence. Such geographic disparities underscore the need for region-specific epidemiological assessments and tailored control strategies.

Ticks responsible for the transmission of *Anaplasma* species are widely distributed across Türkiye and are considered among the most important arthropod vectors. These include genera such as *Ixodes, Haemaphysalis, Dermacentor, Rhipicephalus (Boophilus), Hyalomma,* and *Ornithodorus* [36]. Several studies conducted in this region have documented tick infestations in cattle, sheep, and dogs [17,37-39]. *Dermacentor marginatus* has been reported as a predominant species in both cattle and sheep, with prevalence rates ranging from 18.8% to 66.31% [17,37,38]. Additionally, the presence of *Haemaphysalis parva* (14%-77.27%), *Haemaphysalis punctata* (0.21%-3.0%), *Rhipicephalus bursa* (0.2%-14.62%), *Dermacentor reticulatus* (0.39%-12.5%), and, to a limited extent, *Ixodes ricinus* (0.2%) has been recorded in the same region [17,39,40]. The findings of the present study regarding the presence of *Anaplasma* species in cattle support the hypothesis that the tick species prevalent in Kars, Ardahan, and Iğdır, particularly *Dermacentor marginatus*, play a significant role in the transmission of *Anaplasma* pathogens. The high prevalence of this tick species underscores its potential contribution to the spread of tick-borne diseases in the region.

Anaplasma marginale is recognized as the most prevalent agent of bovine anaplasmosis worldwide, with its biological transmission primarily associated with tick vectors, particularly species from the *Rhipicephalus* and *Dermacentor* genera [41]. In the present study, *A. marginale* was detected in 17.9% of the cattle samples, indicating a relatively high prevalence in the region. These findings suggest that the widespread distribution of *Rhipicephalus* and *Dermacentor* ticks in the study area may contribute significantly to the transmission dynamics of this pathogen. Consequently, effective control of bovine anaplasmosis in the region requires the implementation of integrated tick management strategies tailored to local vector ecology.

In addition to tick vectors, the potential role of other arthropods in the transmission of *Anaplasma marginale* should not be overlooked. Blood-feeding flies such as *Tabanus* spp.[42] and *Stomoxys calcitrans* [43] have been implicated as possible mechanical vectors. These flies may transmit the pathogen by mechanically transferring infected blood between animals. Although ticks remain the principal biological vectors of *A. marginale,* the involvement of hematophagous flies highlights the complexity of its epidemiology. While clinical and field studies consistently demonstrate the effectiveness of tick control strategies, managing fly populations is also essential to reduce the risk of mechanical transmission. Therefore, integrated vector management approaches targeting both ticks and biting flies are recommended for comprehensive disease control.

Although *Ixodes* species are widely recognized as the principal vectors of *A. phagocytophilum* [44], there is ongoing speculation regarding the involvement of other tick species in the transmission of this pathogen [45]. In the present study, the prevalence of *A. phagocytophilum* was found to be 14.00%. Despite the relatively low abundance of *Ixodes* ticks in the study area, the observed infection rate was notably high. This finding raises the possibility that other tick genera may also contribute to the transmission cycle of *A. phagocytophilum* [46]. Previous studies [12,36,47-49] have also supported this hypothesis, suggesting that the presence of *A. phagocytophilum* may not be restricted solely to transmission by *Ixodes ricinus*. These findings underscore the need to consider local tick biodiversity in epidemiological surveillance and control programs. However, further studies are required to confirm the role of alternative vectors. In addition, the results of this study indicate that *A. phagocytophilum* may pose a significant public health risk, not only to cattle but also to humans, particularly those involved in animal husbandry or individuals at increased risk of tick exposure. As a known zoonotic pathogen, its presence in livestock highlights the importance of integrated control strategies to mitigate potential transmission to humans.

Anaplasma bovis is generally associated with subclinical infections; however, it can cause severe disease in immunocompromised animals [50]. In the present study, the highest prevalence of *A. bovis* was recorded in Ardahan province (5.47%), while the lowest was observed in Iğdır (1.77%). These regional differences may be attributed to variations in tick population density and environmental factors. The relatively high prevalence detected in Kars province suggests that local tick species

may act as effective biological vectors for *A. bovis* in this region. Furthermore, the widespread occurrence of subclinical infections implies that many animals may act as asymptomatic carriers, complicating the diagnosis of *A. bovis* in the field and potentially contributing to the silent spread of the pathogen within herds.

The absence of *Ehrlichia* species detection in this study may be attributed to the lack of specific tick vectors belonging to the Ixodid family, particularly *Amblyomma* species, which were not observed in the study region. According to the literature, certain *Ehrlichia* species rely heavily on these tick genera for biological transmission [51] . The absence of such vectors in the current sampling area may explain the failure to detect *Ehrlichia* DNA in the cattle population. This finding suggests that the composition and diversity of the local tick fauna, along with environmental factors influencing vector ecology, may significantly impact the transmission dynamics of *Ehrlichia* spp. Therefore, more comprehensive investigations are needed to clarify the potential presence and epidemiological relevance of *Ehrlichia* species in this region.

The provinces of Kars, Ardahan, and Iğdır are located in the Eastern and Northeastern regions of Türkiye and are characterized by significant ecological diversity. Climatic variations and the presence of extensive pasturelands in these areas play a pivotal role in shaping agricultural and livestock activities. However, these geographical and environmental features also influence the distribution and density of vector populations, particularly ticks, which are responsible for the transmission of various vector-borne diseases. The ecology of each region determines the conditions favorable for tick proliferation, thereby affecting disease dynamics. As such, the geographical and ecological characteristics of each province should be taken into account when designing regional disease control strategies. The climatic and topographic differences among these three provinces likely contribute to the observed variation in the prevalence and transmission routes of *Anaplasma* species. Consequently, the density and distribution potential of tick populations in these regions are critical factors that directly influence the spread of *Anaplasma* spp., highlighting the need for locally tailored control measures to effectively manage these pathogens.

This study has certain limitations. First, it was restricted to three provinces in Northeastern Anatolia, which may limit the generalization of the findings to other regions. Second, sequencing was performed only on a subset of positive samples, meaning that the full genetic diversity of *Anaplasma* spp. in the study area may not have been fully captured. Despite these limitations, the results provide valuable baseline data for future large-scale epidemiological studies.

In conclusion, this study demonstrated the presence of *A. marginale, A. phagocytophilum,* and *A. bovis* in cattle from Northeastern Anatolia, while no *Ehrlichia* spp. were detected. The findings highlight the role of ecological and epidemiological conditions in shaping pathogen distribution and underscore the importance of region-specific control strategies. Although limited to three provinces and partial sequencing, the study provides valuable baseline data for future epidemiological investigations and contributes to the understanding of tick-borne pathogen dynamics in Türkiye.

Declarations

Availability of Data and Materials: The data and materials used in this study are available upon request from the corresponding author (N. Aydın).

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RESEARCH ARTICLE

Molecular Detection and Epidemiology of *Dirofilaria* spp. and *Acanthocheilonema reconditum* in Companion Animals from Central Punjab, Pakistan

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Abstract

Filarial nematodes including *Dirofilaria immitis*, *Dirofilaria repens*, and *Acanthocheilonema reconditum* are vector-borne parasites of veterinary and zoonotic significance, particularly in tropical and subtropical regions. Dogs and cats serve as reservoirs, sustaining parasite transmission in endemic zones. Despite their relevance, these filarial parasites have not been genetically characterized in Pakistan. This study aimed to determine molecular prevalence, assess associated risk factors and perform genetic characterization of *D. immitis*, *D. repens*, and *A. reconditum* in dogs and cats from Faisalabad and Lahore. A total of 400 blood samples were collected from both host species. PCR targeting the SS rRNA gene was performed, followed by sequencing of selected amplicons. Epidemiological data were gathered through structured questionnaires. Sequence analysis was conducted using ClustalX, BioEdit and MEGA X. The overall prevalence was 23.25%, with *A. reconditum* (12.75%) most prevalent and confined to dogs. *D. immitis* (6.5%) and *D. repens* (4.0%) were found in both species, more commonly in dogs. Infections were significantly associated with stray status, outdoor exposure, poor health and vector density. Sequences showed 98-99.5% similarity to global isolates. Phylogenetic analysis confirmed clustering within respective species clades. This study provides the first molecular insight into canine and feline filarial infections in Pakistan and highlights the need for expanded surveillance and vector control strategies.

Keywords: *Dirofilaria* spp., *Acanthocheilonema reconditum*, Epidemiology, Prevalence, Dogs, Cats, Pakistan

INTRODUCTION

Filarial nematodes transmitted by blood-feeding vectors are globally distributed parasites affecting a wide range of mammalian hosts, including domestic animals and humans. In companion animals, filarial infections are often chronic, underdiagnosed and associated with substantial veterinary and public health implications. The transmission and persistence of these parasites are influenced by various factors such as climate, vector density, animal movement and access to veterinary care. In regions with favorable ecological conditions, especially where mosquito vectors thrive, filarial diseases have emerged or re-emerged with increasing frequency [1,2]. Despite their significance, in many endemic regions like Pakistan, data on the true burden of filarial infections in pets remain scarce, particularly using molecular tools that offer superior diagnostic accuracy compared to conventional methods. Among the most clinically important filarial nematodes are *Dirofilaria immitis* and *Dirofilaria repens*, which are responsible for heartworm disease and subcutaneous dirofilariosis, respectively [3]. *D. immitis* resides in the pulmonary arteries and right heart chambers, often leading to severe cardiopulmonary dysfunction in dogs and occasionally in cats. *D. repens*, though less pathogenic, is associated with subcutaneous nodules, dermatitis and conjunctival infections and holds growing zoonotic importance. The distribution of these parasites varies across regions. *D. immitis* is endemic in the Americas, Southern Europe, Southeast Asia and parts of the Middle East, including reports from Iran, Türkiye and India [4,5]. Similarly, *D. repens* has expanded rapidly in Europe with endemicity confirmed in Italy, France, the Balkans and parts of Eastern Europe [6]. In Asia, both parasites have been reported in stray and owned dogs in China, India, Sri Lanka and Thailand, with prevalence

ranging from 2% to over 40% depending on the diagnostic method and host environment [7,8]. However, in Pakistan, published studies remain limited in scope and are largely reliant on microscopy or serological methods, which may underreport true infection rates.

Acanthocheilonema reconditum is another filarial parasite frequently found in dogs, though it is generally considered to be of low pathogenicity. It is transmitted by fleas and lice rather than mosquitoes and is often encountered as a confounding factor in heartworm diagnosis due to overlapping microfilarial morphology. While *A. reconditum* has been detected in countries like Italy, United States, Brazil and Sri Lanka, reports from South Asia remain sporadic and incomplete [9,10]. Although usually asymptomatic, there are increasing reports of co-infection with *Dirofilaria* spp., raising concerns about its epidemiological role and the possibility of misdiagnosis in mixed infections. Molecular tools have become essential to distinguish *A. reconditum* from other filarial species, particularly in endemic regions where mixed infections are likely. In Pakistan, this parasite remains largely neglected in surveillance studies, despite a high population of unmonitored dogs and limited routine veterinary care.

Molecular diagnostics, particularly PCR-based assays, have revolutionized the detection and identification of filarial nematodes by enabling species-level discrimination based on conserved genetic markers such as 18S rRNA and mitochondrial CO1 [9,11]. These tools have revealed previously undetected cases and co-infections in both pets and wildlife, offering greater sensitivity and specificity than microscopy. Risk factor based molecular epidemiological studies are especially valuable as they allow for stratification of prevalence by host, geography, environmental exposure and management practices. The present study aimed to determine the molecular prevalence and associated risk factors of *D. immitis*, *D. repens* and *A. reconditum* in domestic dogs and cats from two major districts of central Punjab, Pakistan, namely Faisalabad and Lahore, using PCR and statistical modeling. This is the first comprehensive investigation combining molecular diagnostics with risk factor analysis for canine and feline filariasis in Pakistan and its findings will contribute significantly to local disease mapping and the development of targeted vector control, preventive management and surveillance strategies.

Material and Methods

Informed Consent and Ethical Approval

Prior to sample collection, informed consent and permission were taken from the respective pet owners, who were briefed about the study objectives. Sampling was carried out under sterile conditions by trained personnel and paramedical staff following ethical and biosafety protocols approved by the Institutional Biosafety and Bioethics Committee (IBC), University of Agriculture, Faisalabad, Pakistan.

Sample Collection

Sampling was conducted in two districts of central Punjab, namely Faisalabad and Lahore, over a two-year period from 2020 to 2022. A total of 400 blood samples were randomly collected from domestic dogs and cats, with an equal distribution of 200 samples per district to ensure adequate spatial representation for epidemiological mapping, rather than species-proportional sampling. A convenience sampling method was employed to obtain 3-5 mL of blood from each animal, targeting both canine and feline populations for the detection of microfilariae. Blood was drawn aseptically from the cephalic vein of stray animals as well as those presented at veterinary hospitals, commercial farms and livestock markets. Each sample was carefully labeled with relevant metadata including host species, date of collection and locality. All blood samples were preserved in EDTA-coated tubes and maintained at 4°C until further processing.

Questionnaire Administration

A structured questionnaire containing open and close-ended questions was used to collect relevant epidemiological information from animal owners and caretakers through interviews. These factors included gender, age, level of outdoor exposure, locality, density of vector breeding sites, availability of veterinary care, living status and health condition of the animals. Locality (urban, peri-urban, rural) was assigned based on the precise sampling site (owner's address or location where the stray animal was caught). The density of mosquito breeding sites was determined individually for each sample through on-site visual assessment and categorized as low, moderate, or high based on the presence of stagnant water, drainage conditions, surrounding vegetation, animal shelters, and owner-reported mosquito nuisance. Questionnaire items were developed after reviewing previous literature and incorporating field insights to ensure contextual relevance.

DNA Extraction and PCR-Amplification

Genomic DNA was extracted from all collected blood samples using the WizPrep gDNA Mini Kit (Wizbiosolutions, Korea), following the manufacturer's standardized protocol to ensure high-quality yield suitable for downstream applications. The extracted DNA was then subjected to polymerase chain reaction (PCR) to amplify the small subunit ribosomal RNA (SS rRNA) gene specific to *Dirofilaria* spp. and *A. reconditum* using newly designed genus and species-specific primer sets. For *Dirofilaria* spp., the primer pair DF: 5'TCGTCATTGCTGCGGTTA-3'

and DR: 3'-TTCGTTTCCGGGAAGCTG-5' was used to amplify a 493bp fragment, while for *A. reconditum*, the primer set ARF: 5'CAGGTGATGGTTTGATGTGC-3' and ARR: 3'-CACTCGCACTGCTTCACTTC-5' targeted a 348bp region. The annealing temperature for both primer sets were optimized at 53°C to ensure specific and efficient amplification. Primer specificity was verified in silico using NCBI Primer-BLAST to ensure correct target binding and to avoid non-specific amplification, and representative PCR products were confirmed by Sanger sequencing.

PCR amplification was carried out in a 20 µL reaction volume consisting of 10 µL of 2X-PCR master mix (Thermo Scientific), 1µL of each forward and reverse primer, 5 µL of template DNA and 3µL of nuclease-free water. The thermal cycling conditions included an initial denaturation at 94°C for 5 min, followed by 40 amplification cycles of denaturation at 94°C for 40 seconds, annealing at 53°C for 30 sec and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR amplicons were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized using a Bio-Rad gel documentation system.

The presence of distinct DNA bands of expected sizes (493bp for *Dirofilaria* spp. and 348bp for *A. reconditum)* confirmed successful amplification. To prepare samples for sequencing, PCR-positive amplicons for each parasite were excised and purified from the gel using the FavorPrep GEL/PCR Purification Kit (Favorgen Biotech, Taiwan) according to the manufacturer's instructions. The purified DNA products were then sent to Lab Genetix, Lahore for paired-end Sanger sequencing to confirm species identity and enable downstream molecular analysis.

Sequence and Phylogenetic Analysis

The obtained nucleotide sequences of *Dirofilaria* spp. and *A. reconditum* were assembled and edited using PREGAP and GAAP4 of staden package (Version 2.0). A nucleotide query for the similarity of related organisms was conducted on NCBI using BLASTn to confirm species identity and determine the closest matching sequences. High-similarity sequences from diverse hosts and geographical locations were subsequently retrieved in FASTA format for downstream analysis. Multiple sequence alignment of the obtained and downloaded sequences was performed using ClustalX software (Version 2.1) [12], allowing identification of conserved regions, point mutations and sequence divergence. Percent nucleotide identity and pairwise evolutionary distances between sequences were calculated using BioEdit software [13], which facilitated assessment of inter and intra-species genetic similarity. Phylogenetic analysis was conducted in MEGA X software using the neighbor-joining method with 1.000 bootstrap replicates and bootstrap values ≥70% were considered to indicate strong node support to infer evolutionary relationships between our sequences and previously published reference sequences.

Statistical Analysis

The association between filarial infection and potential risk factors was analyzed using multiple logistic regression, including age, gender, housing type, outdoor exposure etc. as predictor variables. Moreover, pair-wise odds ratio comparisons were performed between categories within each risk factor at 95% confidence interval using SAS (1998). In addition, to visually represent the spatial and host-related differences in parasite distribution, a heat map was constructed using R package (Version 4.5.1)

Results

Overall Prevalence of *Dirofilaria* spp. and *A. reconditum*

The overall molecular prevalence of filarial parasites in the sampled canine and feline population from central Punjab was 23.25%. Among the three species identified, *A. reconditum* was the most prevalent, followed by *D. immitis* and *D. repens*. Notably, *A. reconditum* was exclusively detected in dogs, with markedly higher prevalence in Faisalabad (30%) than in Lahore (21%). In contrast, *D. immitis* was found in both dogs and cats, with higher detection rates in dogs from Faisalabad (13%) and Lahore (9%) and lower but consistent prevalence in cats (2%) from both districts. *D. repens* showed a more uniform distribution, being detected in both hosts and regions i.e., 5% in dogs and cats from Faisalabad and slightly lower in Lahore with 4% in dogs and 2% in cats. District-wise comparison revealed that filarial infection was more prevalent in Faisalabad, particularly among dogs, which exhibited the highest combined infection rate (18%), followed by dogs from Lahore (13%). Cats showed relatively lower infection rates with 7% positivity in Faisalabad and 4% in Lahore. Overall, the dogs were more affected than cats and Faisalabad exhibited a higher endemicity of filarial parasites than Lahore. These spatial and host-related differences in parasite distribution are visually represented in *Fig. 1*. The heatmap demonstrates clear species-wise clustering by host and district, highlighting the dominant presence of *A. reconditum* in dogs and the comparatively moderate but widespread occurrence of *Dirofilaria* spp. across both hosts and locations. The bar graph alongside further underscores the higher overall burden of *A. reconditum*, indicating its potential epidemiological significance in the studied region.

Risk Factor Analysis

In Faisalabad, the prevalence of *Dirofilaria* spp. was 15.8% in females compared to 9.5% in males, though the difference was not statistically significant (OR=1.781; 95% CI=0.758-4.182). In Lahore, females also had a non-

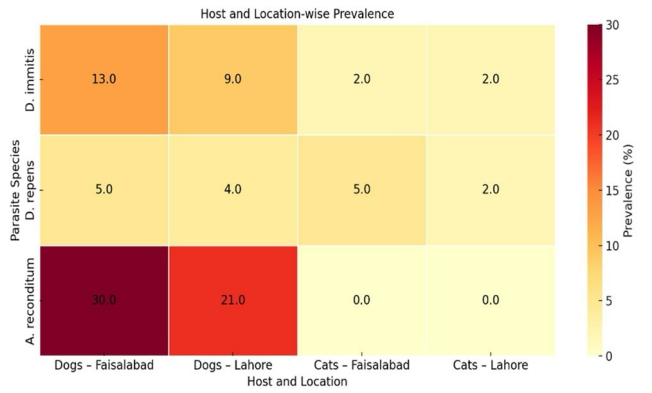

Fig 1. Combined heatmap and bar chart illustrating the prevalence of *D. immitis, D. repens*, and *A. reconditum* in dogs and cats from districts Faisalabad and Lahore, Pakistan. The heatmap shows the percentage of positive samples (n = 100 per group) for each parasite across different host-location combinations. The bar chart represents the overall number of positive cases (n = 400 total samples) for each parasite species

significantly (OR=1.424; 95% CI=0.504-4.0154) higher prevalence (9.6%) than males (7.0%). Age-wise analysis in Faisalabad revealed the highest prevalence in animals older than five years (18.2%; OR=2.444; 95% CI=0.779-7.670), followed by those aged 2–5 years (11.8%) and less than 2 years (8.3%). In Lahore, the same trend was observed with >5 years showing 12.7% prevalence (OR=2.406; 95% CI=0.666-8.685), while 2-5 years and <2 years had 8.0% and 5.7% prevalence, respectively. Outdoor exposure showed a significant association with infection. Animals with high exposure had a prevalence of 20.0% in Faisalabad (OR=3.500; 95% CI=1.072-11.419) and 15.5% in Lahore (OR=3.980; 95%; 95% CI=1.023-15.478). Locality-wise, animals from rural areas showed the highest prevalence in both districts: 20.6% in Faisalabad (OR=3.474; 95% CI=1.177-10.252) and 16.7% in Lahore (OR=3.900; 95% CI=1.107-13.739), while urban and peri-urban animals showed much lower rates. Regarding the density of vector breeding sites, animals from high-intensity areas had significantly higher prevalence in Faisalabad (20.0%; OR=3.813; 95% CI=1.184-12.270), while in Lahore the association was near significant (13.3%; OR=3.692; 95% CI=0.973-14.004). Animals without access to veterinary care were more infected than those with care, showing 18.1% prevalence in Faisalabad (OR=3.277; 95% CI=1.249-8.597) and 14.4% in Lahore (OR=4.474; 95% CI=1.404-14.249). Similarly, stray animals showed significantly higher infection than owned animals, with 18.3% prevalence in Faisalabad (OR=4.524; 95% CI=1.491-13.724) and 12.4% in Lahore (OR=3.215; 95% CI=1.010-10.229). Health status also influenced prevalence, with poor-condition animals showing the highest infection rates of 19.4% in Faisalabad (OR=3.082; 95% CI=0.472-5.228) and 14.0% in Lahore (OR=3.551; 95% CI=1.017-12.397), compared to 7.2% and 4.4% in healthy animals, respectively *(Table 1)*.

In Faisalabad, the prevalence of *A. reconditum* was 17.9% in females and 12.4% in males (OR=1.542; 95% CI=0.705-3.373). In Lahore, females also showed higher prevalence (12.3%) compared to males (8.1%), though the association remained nonsignificant (OR=1.580; 95% CI=0.608-4.102). Regarding age, animals older than five years had the highest prevalence in both districts i.e., 20% in Faisalabad (OR=2.25; 95% CI=0.770-6.569) and 16.4% in Lahore (OR=2.543; 95% CI=0.800-8.086), followed by animals aged 2-5 years (Faisalabad=15.3% and Lahore=9.3%) and <2 years (Faisalabad=10% and Lahore=7.1%). Outdoor exposure was significantly associated with infection in Faisalabad, where animals with high exposure had 24.6% prevalence (OR=3.591; 95% CI=1.225-10.529), while in Lahore the high-exposure group had a near-significant association (17.2%; OR=3.333; 95% CI=0.985-11.273). Locality also played a key role, as rural animals had the highest infection in both Faisalabad (22.1%; OR=3.113; 95% CI=1.130-8.577) and Lahore (20.8%; OR=4.052; 95% CI=1.293-12.693), in contrast to urban and peri-urban areas which showed much lower prevalence.

Animals from areas with high vector breeding intensity had significantly higher prevalence in Faisalabad (22.9%; OR=4.518; 95% CI=1.423-14.345) and Lahore (16.0%; OR=3.381; 95% CI=1.037-11.017), while low-intensity areas had only 6.2% and 5.3% prevalence respectively. Lack of veterinary care was significantly associated with infection, with animals without care showing 20.0% prevalence in Faisalabad (OR=2.388; 95% CI=1.034-5.5157) and 16.7% in Lahore (OR=3.466; 95% CI=1.285-9.350). Stray animals again showed higher infection rates than owned ones i.e., 20% in Faisalabad (OR=2.785; 95% CI=1.134-6.839) and 16.2% in Lahore (OR=4.394; 95% CI=1.422-13.577). Regarding health status, animals in poor condition had significantly higher prevalence of *A. reconditum* in both Faisalabad (25.4%; OR=4.352; 95% CI=1.502-12.606) and Lahore (17.5%; OR=3.659; 95% CI=1.181-11.338), while healthy animals had only 7.2% and 5.5% prevalence, respectively *(Table 2)*.

Sequencing and Phylogenetic Analysis

Pairwise alignment among obtained sequences revealed a high degree of identity within species, ranging from 99.0% to 99.5%, with 2-3 base pair differences. For *D. immitis*, two sequences namely DI-Pak1 (PV848760.1) from cat samples and DI-Pak2 (PV848759.1) from dog samples were analyzed. DI-Pak2 showed the highest similarity (99.58%) with isolates MN795071.1-MN795081.1 from dogs in France and also with Japanese isolates AB973230.1-AB973231.1. It additionally exhibited 99.55% similarity with Iranian isolates (MZ265271.1-MZ265283.1) and 99.12% with a Turkish isolate (PQ496477.1). DI-Pak1, derived from a feline host, showed 99.37% similarity with French and Japanese sequences, followed by 99.33%

Table 1. *Prevalence of Dirofilaria spp. infection in dogs and cats according to different epidemiological risk factors in Faisalabad and Lahore, Pakistan. Values represent number of positive animals out of those tested, prevalence (%), 95% confidence interval (CI), and odds ratio (OR) for the association between each risk factor category and infection status*

Variables	Category	District Faisalabad						District Lahore			
		Positive / Tested	Prevalence (%)	CI (95%)	Odds Ratio	P-Value	Positive / Tested	Prevalence %	CI (95%)	Odds Ratio	P-Value
Gender	Male	10/105	9.5	-	-	-	6/86	7.0	-	-	-
	Female	15/95	15.8	0.758-4.182	1.781	0.185	11/114	9.6	0.504-4.015	1.424	0.504
Age	<2 years	5/60	8.3	-	-	-	4/70	5.7	-	-	-
	2-5 years	10/85	11.8	0.556-1.260	1.474	0.506	6/75	8.0	0.387-5.314	1.435	0.589
	>5 years	10/55	18.2	0.779-7.670	2.444	0.126	7/55	12.7	0.666-8.685	2.406	0.180
Outdoor exposure	Low	4/60	6.7	-	-	-	3/68	4.4	-	-	-
	Medium	8/75	10.7	0.478-5.844	1.672	0.421	5/74	6.8	0.360-6.834	1.570	0.548
	High	13/65	20.0	1.072-11.419	3.500	0.038	9/58	15.5	1.023-15.478	3.980	0.046
Locality	Urban	5/72	6.9	-	-	-	4/82	4.9	-	-	-
	Peri-urban	6/60	10.0	0.431-5.143	1.489	0.529	5/70	7.1	0.386-5.817	1.500	0.558
	Rural	14/68	20.6	1.177-10.252	3.474	0.024	8/48	16.7	1.107-13.739	3.900	0.034
Density of vector breeding site	High	14/70	20.0	1.184-12.270	3.813	0.025	10/75	13.3	0.973-14.004	3.692	0.055
	Moderate	7/65	10.8	0.511-6.619	1.841	0.350	4/50	8.0	0.446-9.754	2.087	0.350
	Low	4/65	6.2	-	-	-	3/75	4.0	-	-	-
Availability of veterinary care	Yes	6/95	6.3	-	-	-	4/110	3.6	-	-	-
	No	19/105	18.1	1.249-8.597	3.277	0.016	13/90	14.4	1.404-14.249	4.474	0.011
Living status	Owned	4/85	4.7	-	-	-	4/95	4.2	-	-	-
	Stray	21/115	18.3	1.491-13.724	4.524	0.008	13/105	12.4	1.010-10.229	3.215	0.048
Health status	Good	5/69	7.2	-	-	-	4/91	4.4	-	-	-
	Fair	7/64	10.9	0.191-1.625	1.572	0.461	5/52	9.6	0.592-9.031	2.314	0.227
	Poor	13/67	19.4	0.472-5.228	3.082	0.044	8/57	14.0	1.017-12.397	3.551	0.047

identity with Iranian isolates (MZ265275.1MZ265283.1) and 98.53% with a mosquito-derived sequence (AF182647.1) from the USA.

For *D. repens*, DR-Pak1 (PV848917.1) from a cat and DR-Pak2 (PV848918.1) from a dog showed strong similarity to various global isolates. DR-Pak2 exhibited 99.58% similarity with sequences from a donkey (MN728180.1) and dog (MN728215.1) in Egypt, as well as with French isolates (MK495734.1, MK495735.1) and a Japanese human-derived isolate (AB973229.1). It also aligned 99.57% with French human-derived isolates (MZ427507.1MZ427510.1). DR-Pak1 showed 99.36% similarity with the same Egyptian isolates and 99.35% with the French human sequences but had lower identity (98.08%) with a Colombian dog isolate (OR029449.1).

For *A. reconditum*, two dog-derived isolates namely AR-Pak1 (PV878140.1) and AR-Pak2 (PV878141.1), were analyzed. AR-Pak1 showed highest similarity (99.03%) with an Indian isolate (GU593976.1) followed by 97.39% identity with a U.S. isolate (MZ468150.1) and 97.06% with Brazilian isolates (KX932116.1, KX932117.1). AR-Pak2 shared 98.70% similarity with the Indian reference sequence and showed lower identity with sequences from the USA (97.06%), Brazil (96.73%), Colombia (MZ473246.1) and Taiwan (AF217801.2).

Phylogenetic trees were constructed for *Dirofilaria* spp. and *A. reconditum* using the neighbor-joining method with 1000 bootstrap replicates. Distant nematodes including *Ascaris* sp. (JN256985.1) and *C. elegans* (EU196001.1) were used as an outgroup in trees to provide stable rooting and preserve correct tree topology. For *D. immitis*, both Pakistani sequences (PV848759.1 and PV848760.1) clustered together in a well-supported clade alongside isolates from Türkiye (PQ496477.1), Iran (MZ265274.1), Japan

(AB973230.1) and France (MZ275161.1), reflecting genetic conservation across Asian and Mediterranean

Table 2. *Prevalence of A. reconditum infection in dogs and cats according to different epidemiological risk factors in Faisalabad and Lahore, Pakistan. Values represent number of positive animals out of those tested, prevalence (%), 95% confidence interval (CI), and odds ratio (OR) for the association between each risk factor category and infection status*

Variables	Category	District Faisalabad					District Lahore				
		Positive / Tested	Prevalence (%)	CI (95%)	Odds Ratio	P-Value	Positive / Tested	Prevalence %	CI (95%)	Odds Ratio	P-Value
Gender	Male	13/105	12.4	-			7/86	8.1	-	-	-
	Female	17/95	17.9	0.705-3.373	1.542	0.277	14/114	12.3	0.608-4.102	1.58	0.347
Age	<2 years	6/60	10.0	-	-	-	5/70	7.1	-	-	-
	2-5 years	13/85	15.3	0.580-4.550	1.625	0.355	7/75	9.3	0.404-4.429	1.338	0.633
	>5 years	11/55	20.0	0.770-6.569	2.25	0.138	9/55	16.4	0.800-8.086	2.543	0.113
Outdoor exposure	Low	5/60	8.3	-	-	-	4/68	5.9	-	-	-
	Medium	9/75	12.0	0.474-4.738	1.5	0.489	7/74	9.5	0.466-5.984	1.671	0.429
	High	16/65	24.6	1.225-10.529	3.591	0.019	10/58	17.2	0.985-11.273	3.333	0.052
Locality	Urban	6/72	8.3	-	-	-	5/82	6.1	-	-	-
	Peri-urban	9/60	15.0	0.648-5.806	1.941	0.235	6/70	8.6	0.421-4.950	1.443	0.559
	Rural	15/68	22.1	1.130-8.577	3.113	0.028	10/48	20.8	1.293-12.693	4.052	0.016
Density of vector breeding site	High	16/70	22.9	1.423-14.345	4.518	0.010	12/75	16.0	1.037-11.017	3.381	0.043
	Moderate	10/65	15.4	0.822-9.349	2.772	0.100	5/50	10.0	0.502-7.736	1.972	0.330
	Low	4/65	6.2	-	-	-	4/75	5.3	-	-	-
Availability of veterinary care	Yes	9/95	9.5	-	-	-	6/110	5.5	-	-	-
	No	21/105	20.0	1.034-5.5157	2.388	0.041	15/90	16.7	1.285-9.350	3.466	0.014
Living status	Owned	7/85	8.2	-	-	-	4/95	4.2	-	-	-
	Stray	23/115	20.0	1.134-6.839	2.785	0.025	17/105	16.2	1.422-13.577	4.394	0.010
Health status	Good	5/69	7.2	-	-	-	5/91	5.5	-	-	-
	Fair	8/64	12.5	0.565-5.912	1.828	0.313	6/52	11.5	0.649-7.750	2.243	0.201
	Poor	17/67	25.4	1.502-12.606	4.352	0.006	10/57	17.5	1.181-11.338	3.659	0.024

Fig 2. Neighbor joining tree showing evolutionary relationship among obtained sequences of SS rRNA gene of *Dirofilaria* spp. with closely related sequences

populations. For *D. repens*, sequences PV848917.1 (cat) and PV848918.1 (dog) formed a tight cluster that aligned closely with the Japanese human isolate AB973229.1 and the Egyptian dog isolate MN728215.1 suggesting intra-species uniformity and potential zoonotic relevance *(Fig. 2)*. In the case of *A. reconditum*, sequences PV878140.1 and PV878141.1 clustered within a distinct, well-supported clade indicating close genetic relatedness and regional specificity. This clade was phylogenetically close to the Indian isolate (GU593976.1) highlighting limited divergence within South Asian populations. Pakistani isolates were clearly distinct from those originating in Brazil, Colombia and Taiwan, which formed separate clades suggesting geographical structuring among global *A. reconditum* lineages *(Fig. 3)*.

Discussion

This study represents the first molecular epidemiological investigation to assess the prevalence and risk factors associated with *Dirofilaria* spp. and *A. reconditum* in domestic dogs and cats from selected districts of central

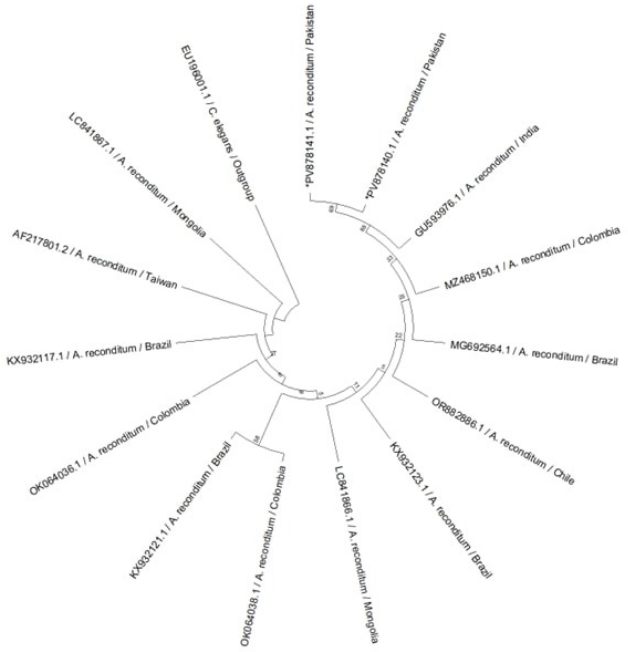

Fig 3. Neighbor joining tree showing evolutionary relationship among obtained sequences of SS rRNA gene of *A. reconditum* with closely related sequences

Punjab, Pakistan. A combined prevalence of 23.25% was recorded for all three filarial species, with *A. reconditum* (12.75%) being the most prevalent, followed by *D. immitis* (6.5%) and *D. repens* (4.0%). Notably, *A. reconditum* was detected exclusively in dogs, whereas *D. immitis* and *D. repens* were found in both dogs and cats, albeit with lower prevalence in feline hosts. This observation aligns with data from the USA and Poland, where *D. immitis* infection is significantly lower in cats, because felines are atypical hosts-larval development is frequently arrested, adult worms have a shorter lifespan, and microfilaremia is transient or absent, leading to reduced detectability and lower overall prevalence [14-16]. Similarly, the higher prevalence of *A. reconditum* and *D. repens* compared to *D. immitis* parallels findings from India, particularly in tropical and subtropical regions where these species dominate [17,18].

Host-related factors showed significant influence on filarial prevalence. Dogs exhibited markedly higher infection rates compared to cats, which reflects their role as primary hosts for *D. immitis* and their increased exposure to vectors due to outdoor activity. Risk was significantly elevated among stray dogs, animals in poor health condition and those lacking veterinary care. These patterns mirror findings from Iran, where *D. immitis* prevalence reached as high as 51.4% among stray dogs in Gilan province, particularly in environments characterized by outdoor exposure, high humidity and insufficient preventive measures [19]. In our study, animals sampled from rural and peri-urban areas with dense mosquito breeding sites showed higher odds of infection, which further supports the role of ecological drivers in transmission.

Environmental and geographical variables emerged as critical determinants of filarial burden. Animals in rural and peri-urban settings were at significantly higher risk compared to urban areas, underscoring the influence of sanitation, vector abundance and access to veterinary care. These findings resonate with global surveillance data from the USA, where the prevalence of *D. immitis* is notably higher in southern regions with warm, humid climates like Texas and Florida, compared to cooler states [14]. In Iran, regional differences in temperature, precipitation and humidity were strongly associated with infection prevalence, highlighted by high rates in Ahvaz and Meshkinshahr and lower prevalence in drier, cooler regions such as Hamadan and Tabriz [19].

Age-wise distribution revealed a trend of increasing infection with age, particularly among adult dogs (>5 years), indicating cumulative vector exposure over time. This is consistent with Iranian studies where dogs aged 3-15 years harbored the highest filarial burdens [20,21]. However, the potential for occult infections, especially with *D. immitis*, must be acknowledged. Up to 30% of dogs may carry adult worms without circulating microfilariae due to pre-patent stages, unisexual infections or immune-mediated clearance [14,17]. This limitation highlights the superior diagnostic accuracy of PCR-based molecular techniques, which were used in the current study and successfully detected infections regardless of blood microfilariae status.

Sequencing of selected PCR-positive amplicons further confirmed species identity and provided insights into genetic similarity and phylogeographic structure. Two *D. immitis* sequences (PV848760.1/cat and PV848759.1/dog) showed 99.58% similarity with French canine isolates (MN795071.1-MN795081.1) and closely clustered with sequences from Iran, Japan and Türkiye in the phylogenetic tree. Similarly, *D. repens* sequences (PV848917.1/cat and PV848918.1/dog) demonstrated 99.58% identity with isolates from Egypt and France and formed a robust clade with human and canine isolates from Japan and Europe, suggesting possible zoonotic potential. For *A. reconditum*, dog-derived sequences (PV878140.1 and PV878141.1) showed highest similarity with an Indian isolate (GU593976.1) and clustered closely in a well-supported clade distinct from those of Brazilian, Colombian and Taiwanese origin. These phylogenetic findings suggest limited intra-species variation within regional populations and underscore the genetic proximity of Pakistani strains to other South Asian isolates.

The phylogenetic analysis based on the SS rRNA gene provided strong support for species level identification,

although limited resolution for intraspecific diversity. High sequence similarity (>99%) within each species and well-supported bootstrap values in NJ trees validated the reliability of our molecular data. However, for finer-scale population genetics and haplotype resolution, future studies should target more variable loci such as ITS2 and CO1 genes. Studies from India have previously shown significant ITS2 variability among *A. reconditum* isolates from different regions [17] suggesting possible cryptic diversity that could also exist in Pakistani populations.

Our results also hold substantial public health relevance due to the zoonotic potential of *D. immitis* and *D. repens*. Human cases of subcutaneous and ocular dirofilariasis due to *D. repens* have been reported in Europe and Asia, often mimicking malignancies and leading to surgical misdiagnoses [6]. Likewise, *D. immitis* has been implicated in pulmonary coin lesions that are radiologically mistaken for tumors, sometimes resulting in unnecessary lung resections [5]. Given the presence of zoonotic species in both feline and canine hosts in our study, regular screening, preventive treatment and mosquito control should be emphasized, especially in endemic regions like central Punjab.

Despite the strengths of molecular confirmation and regionally representative sampling, our study has limitations. The use of convenience sampling may introduce selection bias, and therefore the prevalence estimates should be interpreted cautiously as they may not fully represent the entire companion animal population. The reliance on blood-derived DNA may not detect pre-patent or occult infections with very low parasitemia. Seasonal trends in vector abundance were not tracked and wider geographical surveillance is needed to assess nationwide distribution. Nevertheless, the findings presented here provide the first molecular confirmation of *D. immitis*, *D. repens* and *A. reconditum* in dogs and cats from central Punjab and offer a robust platform for ongoing surveillance, molecular epidemiology and zoonotic risk assessment.

This study provides the first molecular evidence of *D. immitis*, *D. repens* and *A. reconditum* infections in dogs and cats from Pakistan. The overall prevalence of 23.25% highlights the active circulation of filarial parasites in the region, with *A. reconditum* being the most frequently detected species in companion animals. Risk factor analysis revealed that outdoor exposure, rural locality, poor health status, lack of veterinary care and stray living conditions significantly contributed to higher infection rates. These findings emphasize the need for regular screening, improved vector control measures and increased awareness among pet owners and veterinarians to mitigate the spread of these parasites. Given the zoonotic potential of *D. immitis* and *D. repens*, this study also underlines the public health importance of filariasis surveillance and the integration of a One Health approach in endemic regions. Future studies should aim to include larger sample sizes, diverse ecological zones and multilocus molecular analyses to further explore parasite diversity and transmission dynamics in Pakistan.

Declarations

Availability of Data and Materials: All the generated data are included in the manuscript.

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Authors Contributions: MKK: Conceptualization and methodology; ZA: Data Collection, Processing, data analysis, and writing; RZA and MKS: Revised and proof-read. All authors read and approved the final manuscript.

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Research Article

Prognostic Significance of MTDH and Ki-67 Expression in Canine Mammary Tumors: An Immunohistochemical and Survival Study

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Abstract

Biomarkers play critical roles in understanding tumor biology and evaluating prognosis in canine mammary tumor (CMTs) research. MTDH and Ki-67 are crucial factors and markers in the carcinogenesis of multiple organs and tissues in human oncology. However, the role of MTDH in CMTs and its relationship with Ki-67 are not well characterized. This study investigated MTDH and Ki-67 expression and their correlation in 64 benign and malignant CMT tissues using immunohistochemistry (IHC). The association of MTDH and Ki-67 expression with clinicopathological features was also evaluated, followed by assessing their potential prognostic value in a prospective survival study. IHC analysis revealed MTDH expression in both the cytoplasm and nucleus of tumor cells. In contrast, Ki-67 was predominantly in the nucleus. MTDH expression significantly correlated with tumor malignancy grade (P=0.035), tumor size (P<0.0001), Ki-67 index (P<0.0001), and metastasis (P<0.0001). High MTDH expression was significantly associated with reduced disease-free survival (P=0.0042) and overall survival (P=0.0113) in malignant CMTs. These results indicate that the expression levels of MTDH and Ki-67 are positively correlated with adverse clinicopathological parameters and jointly signify aggressive tumor behavior and poor prognosis. MTDH and Ki-67 are thus potential prognostic biomarkers for CMTs.

Keywords: Dogs, Immunohistochemistry, KI-67, Mammary Tumors, MTDH

Introduction

Canine mammary tumors (CMTs) are the most prevalent neoplasms in female dogs, accounting for 70% of all tumors in intact females [1]. They exhibit bimodal distribution and are either benign or malignant. Notably, malignant tumors constitute approximately 50% of CMTs [2]. CMTs exhibit hormone dependency and carry a significant risk of local recurrence post-resection or metastasis, particularly to the lymph nodes and lungs [3]. Mitigating cancer-related morbidity and mortality requires accurate diagnosis and prognostication. Prognostic factors for CMTs, including the histological type, tumor grade, invasiveness, growth rate, lymph node status, and tumor size [4], are crucial for assessing and determining prognosis, as well as predicting tumor molecular behavior.

MTDH (also known as AEG-1 or LYRIC) is a multifunctional oncoprotein strongly associated with initiating breast cancer, metastasis, drug resistance, and immune evasion [5]. It is located within the 8q22 chromosomal region, a frequent site of genomic amplification. Aberrant amplification or transcription at this locus drives MTDH overexpression [6]. This overexpression enhances malignant cell adhesion to circulating blood cells, facilitating tumor metastasis [7]. Noteworthy, MTDH is highly expressed across diverse malignancies [8] but exhibits low expression in non-neoplastic tissues, including normal breast epithelium [9]. It modulates vital signaling pathways such as PI3K/Akt, NF-κB, Wnt/β-catenin, and MAPK [10]. MTDH overexpression in multiple cancer types correlates with critical oncogenic processes including tumorigenesis, proliferation, invasion, metastasis, and chemoresistance [11]. For instance, it promotes tumor growth and proliferation in human breast cancer [12] and further drives invasion, metastasis, and therapeutic resistance [13]. Notably, MTDH has been

identified as a metastasis gene in phage display libraries of metastatic breast cancer, where it binds lung vasculature-associated proteins, mechanistically explaining its role in pulmonary metastasis [14].

Ki-67 is a high-molecular-weight nuclear protein expressed in proliferating cells. It primarily exists as 320 kDa and 359 kDa isoforms [15] and localizes predominantly throughout the nucleoplasm or at the nuclear membrane, serving as a well-established crucial marker of cellular proliferative activity [16]. Ki-67 is expressed during all active phases of the cell cycle: G1, S, G2, and M. However, it is absent in quiescent (G0) cells [17]. The intensity and proportion of nuclear immunoreactivity reflect cellular proliferative activity and aid in evaluating the malignancy potential of neoplasms [18]. Determining Ki-67 protein expression levels in tissues objectively measures the cellular proliferation rate and growth fraction in both tumor and normal tissues. This determination is usually via immunohistochemistry (IHC), and the expression level is typically quantified as the Ki-67 index or proliferation index [19]. In clinicopathological diagnosis, the Ki-67 index is a crucial indicator for tumor grading, aggressiveness assessment, prognostic prediction, and treatment response evaluation in various malignancies, including breast cancer, lymphoma, and neuroendocrine tumors [20]. A high Ki-67 index is generally associated with increased tumor aggressiveness, rapid growth kinetics, and poorer prognosis [21].

This study employed immunohistochemistry (IHC) to detect the expression of MTDH and Ki-67 in canine mammary tumors (CMTs) in dogs. The expression levels were further analyzed to evaluate their relationship and correlate them with clinicopathological features to explore the potential prognostic value of MTDH and Ki-67.

Material and Methods

Ethical Statement

The research protocol used was reviewed and approved by the Research Ethics Committee of Henan Institute of Science and Technology (Approval No: 202009023).

Tissue Samples

A total of 64 surgically resected canine mammary tumor samples and adjacent non-neoplastic tissues were collected from various animal hospitals in Xinxiang City, Henan Province, and the surrounding regions between 2019 and 2023. Histopathological examination confirmed that 30 of the 64 were benign while 34 were malignant neoplasms. All sample collection and usage procedures were performed with informed consent from the pet owners.

This study exclusively included cases with histologically confirmed primary mammary neoplasms following surgical resection. The CMT tissue samples were fixed in 10% neutral-buffered formalin at room temperature for 48 h and subsequently embedded in paraffin blocks. The tissues were then cut into 4 μm-thick sections and stained with hematoxylin and eosin (H&E) for definitive pathological diagnosis. The H&E-stained sections were subsequently mounted on slides and evaluated microscopically. Tumors were classified according to the criteria established by Goldschmidt et al.[22] and histologically graded using the system proposed by Peña et al.[23]. The Ki-67 proliferation index, categorized as ≤15% or >15% positive tumor cells and tumor size categorized as ≤3 cm, 3-5 cm, or >5 cm, were also assessed. The study included cases with solitary and multiple mammary tumors. The tumor exhibiting the most aggressive clinicopathological features was selected for analysis in dogs having multiple malignant tumors [1].

Immunohistochemistry

Paraffin-embedded tissues were cut into sections (4 μm thick) using a rotary microtome (Yidi Medical Equipment, Jinhua, China). The sections were dried at 60°C for 1-2 h, dewaxed by dipping in xylene twice (5 min each), and then rehydrated through a graded ethanol series (100% twice, 95%, 90%, 80%, and 70%; 3 min each). Antigen retrieval was carried out under pressure in citrate-EDTA buffer (pH 6.0; Beyotime Biotechnology, 40xP0086, China) using a DGS-280C pressure cooker (Lichen Technology, China) for 20 min. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide at room temperature for 30 min. Non-specific binding sites were blocked with normal horse serum (Beijing YITA Biotechnology, YT2515, China) for 20 min. The sections were subsequently incubated with primary antibodies overnight at 4°C. The primary antibodies used were goat anti-MTDH (1:300; Jiangsu Qinke Biotechnology, DF13437, China) and rabbit anti-Ki-67 (1:500; Jiangsu Qinke Biotechnology, AF0198, China). The sections were then rinsed with PBS to remove the excess primary antibodies and subsequently incubated for 1 h at room temperature with species-specific HRP-conjugated secondary antibodies. The secondary antibodies used were goat anti-IgG (SOLARBIO Biotechnology, I5256, China) and rabbit anti-IgG (SOLARBIO Biotechnology, SA13, China). The sections were visualized using a DAB chromogen kit (Zhongshan Jinqiao Biotechnology, ZLI-9017, China) for 90 seconds, with the reaction stopped by immersion in distilled water. The sections were counterstained with hematoxylin, dehydrated through graded ethanol, cleared in xylene, and mounted on slides for imaging using a DS-Ri1 microscope (Nikon Corporation, Japan). The sections were rinsed using phosphate-buffered saline (PBS) between all major steps.

Quantitation of IHC Staining

Immunohistochemistry (IHC) results were determined using the immunoreactive score (IRS) method. The IRS method involved the calculation of the immunoreactive score as the product of staining intensity (SI) and the percentage of positive cells (PP). Five random high-power fields (HPFs) were examined under light microscopy in each case, with counts of 100 cells per field. PP was determined as: (the number of positively stained cells/100 cells counted) x 100%. Positive staining for both MTDH and Ki-67 was achieved by the presence of yellow-brown granules within the nucleus or cytoplasm of tumor cells. For mixed histotypes, immunoreactivity was evaluated in the neoplastic epithelial compartment as the primary readout; stromal/mesenchymal staining was documented separately when present. Five representative HPFs (x400) were assessed per case under pathologist guidance.

MTDH expression was immunohistochemically assessed using the Aperio Cytoplasm V2 algorithm, adapted from established human breast cancer criteria [24,25]. Five representative high-power fields (400× magnification) per specimen were analyzed to generate a composite score based on cytoplasmic staining intensity (SI) and percentage of positive tumor cells (PP) [26]. The scoring thresholds were defined as: PP - 0:0%; 1:1-20%; 2: 21-50%; 3: 51-70%; and 4: >70% and SI - 0: no staining; 1: weak (light yellow); 2: moderate (brownish yellow); and 3: strong (dark brown). The immunoreactive score (IRS = PP × SI) categorized the expression as low (IRS<5) or high (IRS≥5). MTDH intensity was manually scored (0-3 scale) [24]. +2 and +3 scores denoted overexpression, while 0 and +1 scores denoted low expression.

Ki-67 immunohistochemical expression was characterized by nuclear-localized brown staining and was quantified using the Aperio Nuclear V9 algorithm [27,28]. This algorithm measured the nuclear reactivity index by calculating the proportion of the positively stained cells as (positive cells/1000 total cells) x 100%. A Ki-67 index ≥15% denoted high proliferative activity regardless of the staining intensity [29]. The CMT cases were stratified into low-risk (<15%) and high-risk (≥15%) prognostic groups through standardized statistical modeling based on this threshold.

Follow-up Data

All dogs with CMTs had follow-up assessments as follows: at least one preoperative visit, every 3 weeks postoperatively for the first 3 months, and quarterly thereafter for ≥2 years. Pet owners were advised to contact the hospital immediately if any abnormalities, including non-CMT-related signs, were observed. All evaluations including physical examinations, thoracic radiography (three views), abdominal ultrasonography, fine-needle aspiration (FNA), biopsy, necropsy (when applicable), and/or computed tomography (CT) when clinically indicated were performed at the Teaching Animal Hospital of Henan Institute of Science and Technology or referred to partner facilities. Newly detected mammary lesions, clinically abnormal lymph nodes, or suspicious lesions in other organs prompted further diagnostic procedures, such as FNA, excisional biopsy, and CT, to exclude secondary tumors or confirm local recurrence or metastasis. All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA).

Statistical Analysis

Case data and diagnostic results from affected dogs were systematically collated. Statistical correlations between MTDH expression and clinicopathological parameters were analyzed using Fisher's exact test, chi-square test, and Pearson/Spearman correlation analysis. Survival outcomes were evaluated by generating Kaplan-Meier curves. Between-group comparisons were assessed via log-rank testing.

Disease-free survival (DFS) was defined as the duration in months from initial surgery to first detection of local recurrence or metastasis. In contrast, overall survival (OS) spanned from surgery to cancer-specific death. The exclusion criteria for OS analysis comprised dogs dying of non-mammary tumor-related causes, dogs lost to follow-up, or those alive at the 24-month endpoint. The exclusion criteria for DFS analysis comprised cases lost to follow-up, dogs that died without metastatic evidence from non-tumor causes, or those that were metastasis-free at 24 months postoperatively. The exclusion timelines on survival curves were denoted by the censored date points. The level of statistical significance was at $P<0.05$.

Results

Data Characteristics

This study comprised 64 histologically confirmed canine mammary tumor (CMT) cases. *Table 1* details the clinicopathological characteristics of all the cases. The mean age of dogs with benign CMTs was 11.00 years (range: 6-16), while that of dogs with malignant cases was 11.94 years (range: 6-15). The cohort included 47 intact and 17 spayed females. The most predominant breeds were Toy Poodles (n=17) and Bichon Frises (n=14). Benign histopathological classifications comprised complex adenomas (n=13), simple adenomas (n=8), and mixed tumors (n=9). Malignant subtypes were identified as mucinous carcinomas (n=14), carcinomas (n=10), carcinosarcomas (n=8), and tubular carcinomas (n=2). Metastatic lesions were confirmed in 4 malignant cases.

Immunolocalization

Immunohistochemical analysis of MTDH and Ki-67 expression across canine mammary tumor subtypes *(Table 2)* revealed distinct localization patterns. MTDH exhibited cytoplasmic and nuclear expression (yellow-brown granules) in neoplastic cells. In contrast, Ki-67 was predominantly in the nuclear (brown granules). Both markers demonstrated significantly higher expression in malignant CMTs compared to benign CMTs. Beyond predominant perinuclear/cytoplasmic staining in neoplastic epithelial cells, clustered stromal/mesenchymal positivity was occasionally observed in mixed malignant subtypes (e.g., carcinosarcoma), consistent with their biphasic composition. Notably, MTDH exhibited heterogeneous stromal distribution in carcinosarcomas and tubular carcinomas. Clustered stromal cells exhibited intensified MTDH immunoreactivity compared to adjacent non-neoplastic tissues *(Fig. 1)*. MTDH positivity rate reached 81%, while high Ki-67 expression (index ≥15%) was observed in 71% of cases among the 64 cases.

Relationship Between MTDH Expression and Tumor Grade or Pathological Factor

There was high MTDH expression in 76.5% of malignant canine mammary tumors (CMTs). High expression rates were observed in 50.0% of grade II and 42.9% of grade III malignancies based on stratification by histological. Elevated MTDH expression occurred in 12.5% of simple adenomas, 15.4% of complex adenomas, and 22.2% of mixed tumors among the benign tumors. Malignant subtypes demonstrated variable expression: 64.3% in mucinous carcinomas, 70.0% in carcinomas, 75.0% in carcinosarcomas, and 50.0% in tubular carcinomas. Notably, MTDH expression levels were significantly correlated with tumor grade (P=0.049), size (P<0.0001), metastatic status (P<0.0001), and Ki-67 index (P<0.0001). However, their levels were not correlated with other clinicopathological parameters, including histological subtype (*Table 3*).

Correlation Between MTDH Overexpression and Clinical Outcome

Kaplan-Meier analysis of the prognostic significance of MTDH overexpression in malignant canine mammary

Table 1. *Comparison of signalment data (age, sex, breed and histologic diagnosis) of benign and malignant mammary gland tumors in 64 dogs*

Characteristic	Benign Tumors (n=30)	Malignant Tumors (n=34)
Median age (range)	11.00 (6-16)	11.94 (6-15)
Sex (n)	Intact female (21) Spayed female (9)	Intact female (26) Spayed female (8)
Breed (n)	Teddy bear dog (10) Bichon Frise (6) Poodle (3) Golden Retriever (3) Cocker spaniel (2) Schnauzer (2) Chow Chow (2) Chihuahua (1) Pekingese (1) Bichon Frise (1)	Bichon Frise (8) Teddy bear dog (7) Poodle (5) Golden Retriever (4) Schnauzer (4) Cocker spaniel (2) Alaskan malamute (2) Pekingese (1) Chihuahua (1) Bichon Frise (1)
Histologic type (n)	Complex adenoma (13) Adenoma simplex (8) Mixed adenoma (9)	Myxoid fibroma (14) Adenofibroma (10) Carcinosarcoma (8) Tube-like tumor (2)

Table 2. *Statistical results of immunohistochemical examination of canine mammary tumors*

Type of Tumor	Classification	The Expression Status of MTDH	The Expression Status of Ki-67	P
Benign tumor	Adenoma simplex	Weakly positive (+)	Weakly positive (+)	<0.001
	Complex adenoma	Weakly positive (+)	Weakly positive (+)	
	Mixed adenoma	Weakly positive (+)	Weakly positive (+)	
Malignant tumor	Myxoid fibroma	Positive (++)	Weakly positive (+)	<0.001
	Adenofibroma	Positive (++)	Weakly positive (+)	
	Carcinosarcoma	Positive (++)	Positive (++)	
	Tube-like tumor	Strongly positive (+++)	Strongly positive (+++)	

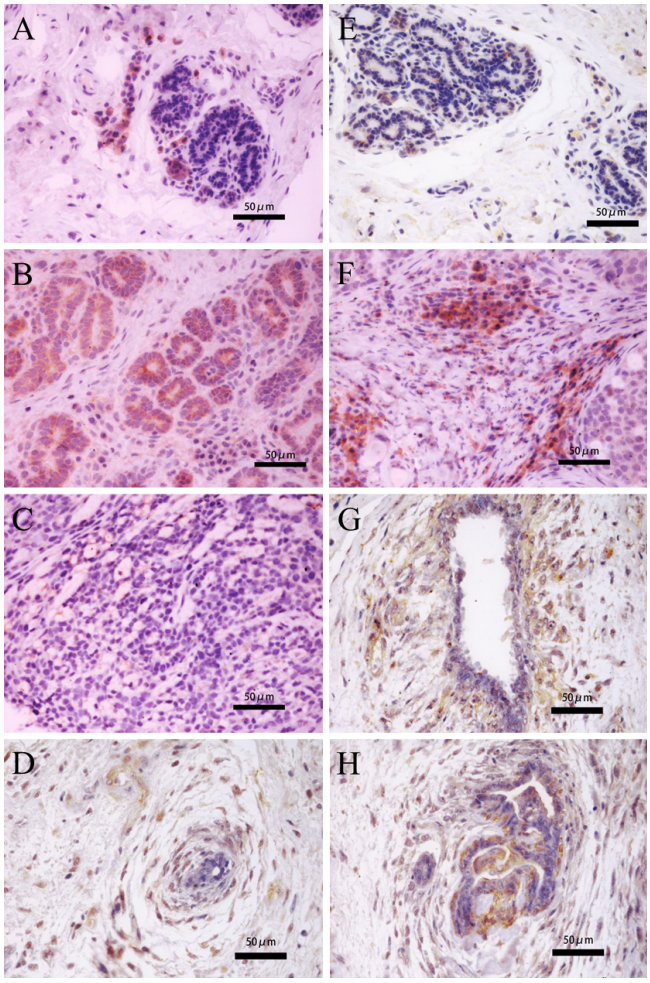

Fig 1. Immunohistochemical localization of MTDH and Ki-67 in canine mammary tissues. MTDH shows predominant perinuclear/cytoplasmic staining in neoplastic epithelial cells, whereas Ki-67 displays nuclear labeling. MTDH panels: (A) non-neoplastic mammary tissue, negative (–); (B) benign mammary tumor, weak (+); (C) carcinosarcoma, moderate (++); (D) tubular carcinoma, strong (+++). Ki-67 panels: (E) non-neoplastic mammary tissue, negative (–); (F) benign mammary tumor, weak (+); (G) carcinosarcoma, moderate (++); (H) tubular carcinoma, strong (+++). All sections are counterstained with hematoxylin; objective magnifications and scale bars are indicated

Table 3. *Expression of MTDH and KI-67 in canine mammary tumors and analysis of their relationship with histological grading, clinical staging and characteristics*

Variable		MTDH Expression			P
		Number of Tumors	Low	High	
Benign tumor	Adenoma simplex	8	7	1	0.854
	Complex adenoma	13	11	2	
	Mixed adenoma	9	7	2	
Malignant tumor	Myxoid fibroma	14	5	9	0.991
	Adenofibroma	10	3	7	
	Carcinosarcoma	8	2	6	
	Tube-like tumor	2	1	1	
Histological grade	I	8	4	4	0.049
	II	12	5	7	
	III	14	1	13	
Tumor size	<3 cm	8	8	0	<0.0001
	3~5 cm	8	0	8	
	>5 cm	48	16	32	
Metastases	Absent	14	14	0	<0.0001
	Present	20	0	20	
KI-67 labelling index	≤15%	14	12	2	<0.0001
	>15%	50	12	38	

tumors (CMTs) revealed that there were 21 tumor-related deaths with 8 censored observations among the 34 malignant cases. Survival curves demonstrated significantly reduced disease-free survival (DFS; median=18 months) and overall survival (OS; median=24 months) in the high-expression (n=26) cohort (log-rank P<0.05) compared to the low-expression (n=8) cohort. Noteworthy, dogs with MTDH overexpression exhibited worse prognoses than their low-expression counterparts across both survival endpoints.

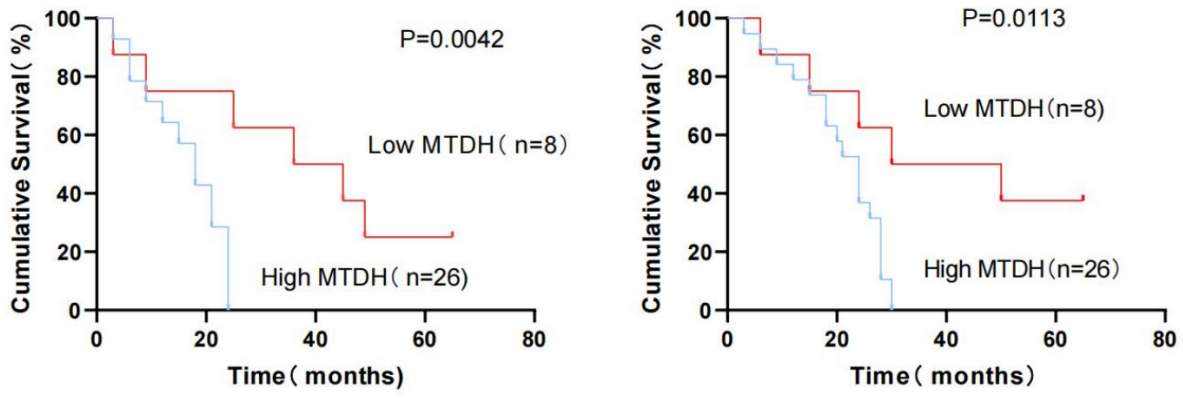

Fig 2. Kaplan-Meier survival curves of 34 dogs with malignant CMTs based on MTDH expression status for A, disease-free survival (median: 18 months) and B, overall survival (median 24 months)

Discussion

This study investigated MTDH and Ki-67 expression in canine mammary tumors (CMTs). MTDH demonstrates negligible expression in normal tissues. However, it is overexpressed in diverse malignancies, including mammary carcinoma. Mechanistically, MTDH enhances tumor-endothelial adhesion, facilitating vascular invasion and distant colonization [30]. Human oncology studies [31] postulate that elevated MTDH levels correlate with advanced tumor stage, lymph node metastasis, and poor prognosis in CMTs. This correlation is potentially through pro-proliferative and anti-apoptotic pathway activation [32]. Notably, MTDH exhibits preferential expression at tumor invasion fronts, corroborating its prometastatic role. MTDH overexpression significantly reduces the overall survival (OS) and disease-free survival (DFS) in human cancers, including breast, ovarian, and pancreatic cancers [33]. The findings of this study confirmed parallel prognostic implications in CMTs, highlighting MTDH as a critical regulator of cell cycle progression and proliferation. Functionally, MTDH drives metastasis, mediates therapeutic resistance, and maintains cancer stemness, positioning it as a molecular linchpin in CMT malignant progression [34]. Herein, MTDH expression was significantly associated with the pathological grade, metastatic risk, and survival outcomes, conferring dual utility as a diagnostic biomarker and therapeutic target [35]. Emerging human-targeted agents, such as C26-A6 inhibitors and MitoQ, offer translational potential for canine oncology. However, direct veterinary clinical evidence remains limited [36].

The associations between MTDH expression and clinicopathological parameters, including tumor size, histologic subtype, grade, metastasis, and Ki-67 index, highlighted MTDH expression as a prognostic indicator in canine mammary tumors (CMTs) [37]. The higher MTDH overexpression observed in 'connective tissue–associated' tumors reflect the biology of mixed malignant histotypes (e.g., carcinosarcoma) rather than contradicting the overall increase seen in malignancies. These entities contain variable epithelial and mesenchymal proportions that can modulate apparent immunoreactivity on IHC. While our primary objective was to evaluate overall prognostic associations, we acknowledge that compartment-level heterogeneity may confound pooled comparisons. Future studies using dual-marker IHC (e.g., cytokeratin/vimentin) and compartment-aware digital quantification are warranted to delineate cell-type-specific MTDH expression and refine prognostic modeling. MTDH overexpression significantly correlated with advanced tumor grade, metastatic dissemination, and elevated Ki-67 expression. Ki-67 levels were significantly higher in malignant CMTs than in benign CMTs, consistent with human oncology paradigms. Notably, MTDH exhibited a strong positive correlation to Ki-67, highlighting MTDH's involvement in proliferation-driven tumor progression. Mechanistically, MTDH activates epidermal growth factor receptor (EGFR), triggering MAPK/ERK pathway signaling that upregulates cyclin D1 expression. The upregulation of cyclin D1 accelerates G1-S phase transition and enhances proliferative capacity [18]. MTDH also inhibits apoptosis by activating PI3K/AKT, further augmenting tumor cell accumulation [18]. These synergistic pathways functionally converge with Ki-67 overexpression to potentiate neoplastic growth [38]. Clinically, dual assessment of MTDH/Ki-67 provides superior prognostic stratification compared to single-marker evaluation. CMTs exhibiting co-expression of both markers exhibit higher invasiveness and recurrence risk, warranting intensified adjuvant therapy [39]. However, these biomarkers should be validated against standardized clinical endpoints, including tumor burden, nodal status, and survival, to establish evidence-based implementation protocols using multi-institutional studies.

This study had several methodological limitations. A limitation of this study is reliance on manual, light-microscopy-based semiquantitative scoring (IRS), which may introduce observer subjectivity in densely stained regions. Future studies will prospectively incorporate standardized digital image analysis in an independent cohort to validate and refine these IRS-based estimates. While the sample size of 64 cases is generally acceptable for a veterinary pathology study, the unbalanced distribution of benign (n=30) and malignant (n=34) cases may limit the statistical power and generalizability of our findings. The unequal distribution could introduce biases, particularly when evaluating prognostic factors that may vary between tumor subtypes. Moreover, the limited incidence of MTDH overexpression in benign tumors (≤22.2% across subtypes) hindered robust correlation assessments between histopathological classifications and clinicopathological features. Additionally, the study did not include translational validation of MTDH expression through quantitative methods, such as mRNA quantification (RT-qPCR) or protein immunoblotting (Western blot). Future studies with larger, more balanced cohorts and molecular profiling to elucidate MTDH's regulatory dynamics in both physiological and neoplastic contexts are necessary to confirm the applicability of these results to a broader population of canine mammary tumors and to ensure more reliable prognostic interpretations.

The findings of this study collectively establish foundational evidence for evaluating MTDH expression and its association with Ki-67 in canine mammary tumors. The dual-marker assessment paradigm demonstrates significant potential as a cornerstone for precision

oncology in CMTs management. The findings herein substantiate the prognostic utility of MTDH and Ki-67 and advocate for further investigation into their dual functionality as diagnostic biomarkers and therapeutic targets in translational veterinary oncology.

Declaration

Availability of Data and Materials: The data and materials used in this study are available upon request from the corresponding author (X. Xia).

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Research Article

The Role of Serum Endocan as a Prognostic Biomarker in Calves with Enzootic Pneumonia

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Abstract

Respiratory system infections are one of the most critical problems that can cause serious economic losses and mortality in cattle breeding. Our study aimed to investigate the significance of endocan levels, which have been shown to yield successful outcomes in predicting prognosis in various respiratory disorders in human medicine, as a reliable prognostic biomarker in calves affected by enzootic pneumonia (EP). The study also examined the efficacy of lactate levels and a modified respiratory scoring system (MSS) in predicting death, alongside endocan levels. Calves with EP (n=53) and healthy calves (n=27) were included in the study and only single blood sample was collected from each calf in the study. Blood samples were taken from the EP group prior to treatment. Our investigation revealed that endocan levels were considerably lower in calves with EP compared to healthy calves (P<0.001). Our study assessed the diagnostic and prognostic significance of endocan and lactate levels in calves with EP using ROC analysis. The cut-off values for endocan and lactate employed in illness prediction were <70.41 (AUC=0.734, P=0.001) and >2.55 (AUC=0.842, P<0.001), respectively. Cut-off values for mortality prediction were >64.96 for endocan (AUC=0.657, P=0.052), >3.90 for lactate (AUC=0.940, P<0.001), and >7.5 for MSS (AUC=0.848, P<0.001). We concluded that endocan had limited diagnostic utility. However, the modified scoring system showed considerable efficacy in predicting mortality. Furthermore, lactate levels have been shown to exhibit superior accuracy and clinical value for both diagnosis and prognosis.

Keywords: Endocan, Enzootic pneumonia, Lactate, Modified respiratory scoring system, Prognostic biomarker

Introduction

Respiratory tract infections are the second most significant cause of calf mortality [1,2]. Bacterial, viral, and parasitic agents are primarily involved in respiratory diseases of calves [2]. In addition to these pathogens, predisposing factors such as poor nutrition and housing conditions, primary diseases, transportation stress, and adverse weather conditions play a critical role in the onset of disease [2,3]. Affected animals act as important sources of transmission and shed large quantities of viruses and bacteria through nasal discharge. Clinical signs of the disease include fever, nasal discharge, coughing, dyspnea, anorexia, depression, lethargy, and death.

The diversity of causative agents, high morbidity and mortality rates, antimicrobial resistance, and diagnostic limitations in field conditions delay the establishment of effective treatment protocols, resulting in significant calf losses. Furthermore, the disease imposes additional labor and economic burden on farms due to treatment time and medication costs. Therefore, prompt and accurate determination of treatment protocols and prognosis is essential to minimize these costs [2,4]. In this context, the identification and development of novel biomarkers for the diagnosis of respiratory diseases and their transformation into applicable preparations for use in veterinary practice are of great importance for the sustainability of the livestock industry.

Previously known as endothelial cell-specific molecule-1 (ESM-1), endocan is a 50 kDa proteoglycan secreted by various organs, including vascular endothelial cells, cardiomyocytes, lungs, skin, gastrointestinal tract, liver, brain, lymph nodes, and kidneys [5-8]. In humans, it is detectable in both urine and plasma and has been suggested as a useful biomarker for monitoring and predicting the course of various diseases due to its low concentration

and high stability under physiological conditions [7]. In human medicine, endocan has shown promising results in predicting the prognosis of inflammatory conditions such as acute respiratory distress syndrome (ARDS) and hospital-acquired pneumonia [5,6,9]. It has also been reported that endocan modulates several biological processes, including cell proliferation, neovascularization, and cellular adhesion, owing to its ability to interact with bioactive proteins [8]. The prognostic value of endocan has been demonstrated in inflammatory disorders, tumor progression, sepsis, hypertension, diabetes, cardiovascular diseases, and chronic kidney disease. A positive correlation has been reported between endocan levels and cardiovascular risk factors such as hypertension, diabetes mellitus, and chronic renal failure [8,10]. Lassalle et al.[11] found that the secretion of endocan into the bloodstream is upregulated by pro-inflammatory cytokines, particularly interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) [12-14].

This study evaluated the potential of endocan levels as prognostic biomarkers in calves with enzootic pneumonia (EP), based on its proven prognostic value in inflammatory diseases such as ARDS and hospital-acquired pneumonia in human medicine.

Material and Methods

Ethical Statement

This study was conducted with the permission of the Erciyes University Animal Experiments Local Ethics Committee (ERUHADYEK) (Approval date and no: 07.06.2023/128)

Animals

A total of 80 calves, including EP (n=53) and healthy (n=27) calves brought to Erciyes University, Faculty of Veterinary Medicine, Department of Internal Medicine, Ruminant Clinic for examination and general control, were included in our study.

Inclusion and Exclusion Criteria

Animals participating in the study were enrolled after obtaining informed, voluntary consent from their owners and meeting the predefined inclusion and exclusion criteria. The study involved calves aged 2-6 months. Clinically healthy calves with no abnormal findings and a total clinical score of 0 were included as the control group. Calves showing clinical signs consistent with respiratory disease and with a total clinical score ≥1 were included in the EP group.

Calves presenting with symptoms such as cough, nasal discharge, and pathological lung sounds were included in the study, whereas those with congenital anomalies, enteritis, omphalitis, arthritis, other disease symptoms, or a history of prior treatment were excluded.

Clinical Examination and Modified Scoring System

The clinical examinations of the patients presenting at the Veterinary Faculty Education, Research, and Application Hospital were conducted systematically. Vital signs were measured in all calf groups before sample collection. Findings obtained through clinical and laboratory evaluations were recorded and assessed. In addition to clinical examination, a modified clinical respiratory scoring system (MSS) was applied to sick calves for prognostic purposes by modifying the scoring systems used by Hägglung et al. [15] and Love et al. [16]. In this modified score system, clinical symptoms including rectal temperature (°C) [0 point (37.7 - 37.8), 1 point (<37.7 or >37.8)-2 point (<36 or >39.2)], respiratory rate (breath/min) [0 point (36 - 60), 1 point (<36 or >60), 2 point (<24 or >80)], pulse rate (bpm) [0 point (100-120), 1 point (<100 or >120), 2 point (<80 or >140)], nasal discharge [0 point (normal), 1 point (serous), 2 point (seromucous, mucopurulent and purulent)], cough [0 point (no cough), 1 point (rare sporadic cough), 2 point (cough at least once every 10 minutes while the calf is at rest)], lung auscultation sound [0 point (no abnormal sounds), 1 point (pathological sounds, crackles, whistles etc.)] and general condition [0 point (lively, active), 1 point (mildly-depressed), 2 point (moderate to severe depression)] were scored between a minimum of 0 and a maximum of 13.

Similar treatment protocols were applied to all sick calves to ensure uniformity in the study. The owners were contacted 15 days after the calves were discharged, and information about the prognosis of the calves was obtained.

Hemogram and Blood Gases Analysis

Only single blood sample was collected from each calf in the study. Blood samples were taken from the EP group prior to treatment. Blood samples of 4 mL in EDTA K3 tubes, 8 mL in gel tubes, and 1.5 mL in blood gas syringes were taken from the calves' vena jugularis once. Blood samples taken for ELISA analysis were centrifuged at 3000 rpm for 20 min to separate the serum.

Collected samples for hemogram and blood gas analysis were processed within the 15 min following blood-letting. Complete blood count [(Leukocyte (WBC), Lymphocyte (LYM), Monocyte (MON), Granulocyte (GRA), Erythrocyte (RBC), Hemoglobin (HGB), Hemotocrit (HCT) and Platelet (PLT)] was carried out in Exigo Eos device (Haematology analyzer, Boule Medical, Sweden) on K_3 EDTA venous blood samples collected from all calves. Blood gases analysis [pH, pCO_2 (mmHg), pO_2 (mmHg), Potassium, sodium, chloride, calcium, Lactate, bicarbonate, and anion gap (AGP)] was carried out in ABL 80 FLEX (Blood Gas/Electrolyte Analyzer) on the heparinized venous blood samples taken from all calves.

ELISA Analysis

In the collected serum samples, serum endocan levels were measured using the Bovine Endocan ELISA kit (SunRed Biotechnology Company, Cat No: 201-04-4681, Assay range: 5-1500 ng/L, Intra-Assay: CV<10%, Inter-Assay: CV<12%) and the proinflammatory cytokines IL-1β (SunRed Biotechnology Company, Cat No: 201-04-0157, Assay range: 1. 5-400 pg/mL), TNF-α (SunRed Biotechnology Company, Cat No: 201-04-0007, Assay range: 15-4000 ng/L, Intra-Assay: CV<9%, Inter-Assay: CV<11%) and IL-6 (SunRed Biotechnology Company, Cat No: SRB-T-83200, Assay range: 30-6000 ng/L, Intra-Assay: CV<10%, Inter-Assay: CV<12%) Bovine ELISA kits were analyzed in accordance with the ELISA protocol of the kits.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA). The normality of the data was evaluated by histograms, Q-Q plots, and the Shapiro-Wilk test. Independent sample t-test (alternative: Mann-Whitney U Test) was used for intergroup comparisons. Pearson correlation analysis was performed on parameters obtained from all animals included in the study (n=80) to determine the direction and strength of the relationship between the variables. ROC (Receiver Operating Characteristic) analysis was performed to evaluate the diagnosis (EP vs healthy calves) and prognosis (died calves with EP vs survived calves with EP) between the groups. Data were expressed as mean±standard deviation and median (min-max). P<0.05 was considered statistically significant.

Results

Of the 80 calves included in the study, 85% (n=68) were Simmental, 6.25% (n=5) were Holstein, 6.25% (n=5) were Montafon, and 2.5% (n=2) were other breeds. 58.8% (n=47) of the calves were male and 41.2% (n=33) were female. 55% (n=52) of the calves were 2 months old and 35% (n=28) were 3-6 months old.

All healthy calves (n=27) were 2 months old and of Simmental breed. Of these calves, 51.9% (n=14) were male and 48.1% (n=13) were female.

Of the calves with EP (n=53), 77.4% (n=41) were Simmental, 9.4% (n=5) were Holstein, 9.4% (n=5) were Montafon, and 3.8% (n=2) were other breeds. Of these calves, 52.8% (n=28) were 3-6 months old and 47.2% (n=25) were 2 months old. Of the calves with EP, 62.3% (n=33) were male and 37.7% (n=20) were female. It was learned that 56.6% (n=30) of the calves with EP were alive and 43.4% (n=23) died.

Statistically significant differences were found in mean rectal temperature and respiratory rate between healthy calves and calves with EP (P<0.001, respectively). Mean rectal temperature (38.1±0.6°C) and respiratory rate (37.6±8.9) in healthy calves were lower than mean rectal temperature (38.9±1.1°C) and respiratory rate (68.6±15.6) in calves with EP. No statistically significant difference was found in mean pulse rate between healthy calves (114.3±9.9 bpm) and calves with EP (107.5±30.3 bpm) (P=0.141).

There was no statistically significant difference between the rectal temperature variables of deceased (39.5±1.5°C) and surviving (38.7±0.8°C) calves with EP (P=0.314). However, there was a statistically significant difference between the respiratory rate and pulse rate variables of deceased and surviving calves (P=0.001, P=0.038, respectively). The respiratory rate (81.5±25.5) and pulse rate (117.3±31.4) of deceased calves with EP were higher than the respiratory rate (58.6±21.2) and pulse rate (99.9±27.7) of surviving calves *(Table 1)*.

In the MSS performed on calves with EP, a statistically significant difference was identified found between the scores of calves that died and survived (P<0.001). The mean score of calves that died with EP (8.74±2.59) was higher than the score of calves that survived (5.13±2.59).

A comparison of the hemogram findings of healthy and EP calves revealed a statistically significant difference in the variables WBC (10^9/L), LYM (10^9/L), GRA (10^9/L), RBC (10^{12}/L), HGB (g/dL), HCT (%) and PLT (10^9/L) (P<0.001, P=0.004, P=0.022, P=0.002, P=0.008, P=0.042, P=0.013, respectively). A statistically significant difference was identified between the WBC (10^9/L), LYM (10^9/L), GRA (10^9/L), RBC (10^{12}/L), HGB (g/dL), HCT (%), and PLT (10^9/L) variables of deceased and surviving calves with EP (P=0.002, P=0.014, P=0.049, P=0.006, P=0.03, P=0.026, P=0.022, respectively) *(Table 2)*.

Table 1. *Vital signs values of calves that died and survived with enzootic pneumonia and healthy calves*

Variable	Healthy Calves (n=27)	Enzootic Pneumonia (n=53)	P
Rectal temperature (°C)	38.1±0.6	38.9±1.1	<0.001
Respiratory rate (min)	37.6±8.9	68.6±15.6	<0.001
Pulse rate (bpm)	114.3±9.9	107.5±30.3	0.141
Variable	**Died Calves (n=23)**	**Survived Calves (n=30)**	**P**
Rectal temperature (°C)	39.1±1.5	38.7±0.8	0.314
Respiratory rate (min)	81.5±25.5	58.6±21.2	0.001
Pulse rate (bpm)	117.3±31.4	99.9±27.7	0.038

Data were expressed as mean±standard deviation. The result is statistically significant at the P<0.05 level

Table 2. *Hematological and blood gases values of calves that died and survived with enzootic pneumonia and healthy calves*

Parameter	**Variable**	**Healthy Calves (n=27)**	**Enzootic Pneumonia (n=53)**	**P**
Hematological Changes	WBC (10^9/L)	7.60 (4.60-13.00)	10.50 (3.40-61.70)	<0.001
	LYM (10^9/L)	2.50 (0.30-5.50)	3.50 (0.60-13.00)	0.004
	MON (10^9/L)	1.00 (0.40-1.70)	0.90 (0.30-10.10)	0.543
	GRA (10^9/L)	4.40 (1.10-8.60)	5.90 (1.40-38.60)	0.022
	RBC (10^{12}/L)	6.22±1.01	7.43±2.14	0.002
	HGB (g/dL)	8.59±1.59	9.94±2.40	0.008
	HCT (%)	24.35±4.11	26.93±7.21	0.042
	PLT (10^9/L)	499.00 (50.00-1308.00)	321.00 (50.00-1527.00)	0.013
	Variable	**Died Calves (n=23)**	**Survived Calves (n=30)**	**P**
	WBC (10^9/L)	12.00 (3.40-23.80)	10.50 (6.20-61.70)	0.002
	LYM (10^9/L)	3.40 (0.90-5.90)	3.50 (0.60-13.00)	0.014
	MON (10^9/L)	0.80 (0.30-1.90)	1.00 (0.30-10.10)	0.555
	GRA (10^9/L)	5.45 (1.40-17.90)	7.40 (1.90-38.60)	0.049
	RBC (10^{12}/L)	7.71±2.22	7.22±2.09	0.006
	HGB (g/dL)	10.16±2.39	9.78±2.44	0.03
	HCT (%)	28.86±7.61	25.45±6.63	0.026
	PLT (10^9/L)	459.00 (50.00-1527.00)	275.00 (50.00-1217.00)	0.022
Blood Gases Changes	**Variable**	**Healthy Calves (n=27)**	**Enzootic Pneumonia (n=53)**	**P**
	pH	7.38±0.05	7.37±0.11	0.551
	pCO_2 (mmHg)	39.49±2.09	43.17±8.79	0.005
	pO_2 (mmHg)	28.93±1.92	32.60±6.03	<0.001
	Na (mmol/L)	141.15±1.75	135.70±4.15	<0.001
	K (mmol/L)	4.54±0.34	4.23±0.74	0.01
	Ca (mmol/L)	1.31±0.02	1.19±0.09	<0.001
	Cl (mmol/L)	95.74±2.16	95.14±4.73	0.443
	Lactate (mmol/L)	2.41±0.49	3.98±1.64	<0.001
	HCO_3 (mmol/L)	32.84±1.77	27.31±4.98	<0.001
	BE (mmol/L)	2.40 (-1.30-9.00)	2.90 (-20.30-10.70)	<0.001
	AGP (mmol/L)	16.66±2.25	20.16±5.29	<0.001
	Variable	**Died Calves (n=23)**	**Survived Calves (n=30)**	**P**
	pH	7.34±0.15	7.39±0.06	0.082
	pCO_2 (mmHg)	43.01±8.74	43.29±8.96	0.910
	pO_2 (mmHg)	30.65±6.35	34.10±5.40	0.038
	Na (mmol/L)	136.00±4.57	135.47±3.87	0.648
	K (mmol/L)	4.19±0.94	4.26±0.56	0.735
	Ca (mmol/L)	1.21±0.08	1.18±0.09	0.340
	Cl (mmol/L)	96.38±4.38	94.27±4.86	0.118
	Lactate (mmol/L)	5.34±1.31	2.93±0.96	<0.001
	HCO_3 (mmol/L)	26.21±5.33	28.15±4.61	0.162
	BE (mmol/L)	3.10 (-20.30-7.30)	2.55 (-7.00-10.70)	<0.001
	AGP (mmol/L)	21.01±6.70	19.57±4.06	0.346

Data were expressed as mean±standard deviation. Data that did not provide normality distribution were expressed as median (min-max). EP: Enzootic pneumonia, WBC: Total leukocytes, LYM: Lymphocyte, MON: Monocyte, GRA: Granulocyte, RBC: Erythrocyte, HGB: Hemoglobin, HCT: Hematocrit, PLT: Platelets, pCO_2: Partial pressure of carbondioxide, pO_2: Partial pressure of oxygen, BE: Base excess, AGP: Anion gap

Significant differences were found in blood gas and electrolyte parameters [pCO_2, pO_2, Na^+, K^+, Ca^{2+}, lactate, HCO_3^-, base excess (BE), and anion gap (AGP)] between healthy and EP calves (all P<0.001, except for K^+, P=0.01 and pCO_2, P=0.005). When blood gas findings of deceased and surviving calves with EP were compared, a statistically significant difference was found for pO_2, lactate, and BE variables (P=0.038, P<0.001, P<0.001, P<0.001, respectively) *(Table 2)*.

Serum endocan levels were found to be significantly lower in calves with EP compared to healthy calves (P<0.001). Serum TNF-α levels were significantly higher in calves with EP than in healthy calves (P=0.022). Serum IL-1β levels were significantly higher in healthy calves compared to those with EP (P=0.004). No statistically significant difference was observed in serum IL-6 levels between calves with EP and healthy calves (P>0.05) *(Table 3)*. A

Table 3. *Endocan and other cytokine levels in calves with enzootic pneumonia and healthy calves*

Parameter	**Variable**	**Healthy Calves (n=27)**	**Enzootic Pneumonia (n=53)**	**P**
Comparison of diseased and healthy calves	Endocan (ng/L)	80.88±19.02	63.58±18.61	<0.001
	TNF-α (ng/L)	234.49±69.91	281.72±109.92	0.022
	IL-1β (pg/mL)	37.74±12.36	29.45±11.69	0.004
	IL-6 (ng/L)	313.03±90.17	335.38±110.42	0.367
Parameter	**Variable**	**Died Calves (n=23)**	**Survived Calves (n=30)**	**P**
Comparison of died and survived calves	Endocan (ng/L)	69.60±18.99	58.97±17.23	0.038
	TNF-α (ng/L)	375.82±83.55	209.58±62.95	<0.001
	IL-1β (pg/mL)	34.30±12.65	25.73±9.52	0.007
	IL-6 (ng/L)	414.08±101.94	275.05±72.82	<0.001

Data were expressed as mean±standard deviation. The result is statistically significant at the P<0.05 level, TNF-α: Tumor necrosis factor alfa, IL-1β: Interleukin 1 beta, IL-6: Interleukin 6

Table 4. *Predictive/prognostic value of serum endocan and lactate for enzootic pneumonia*

Predictive	**n**	**Parameters**	**Cut-off**	**AUC (95% CI)**	**Se (%)**	**Sp (%)**	**P**
EP	53	Endocan (ng/L)	<70.41	0.734 (0.621-0.847)	67	64	0.001
		Lactate (mmol/L)	>2.55	0.842 (0.754-0.931)	83	82	<0.001
Mortality	23	Endocan (ng/L)	>64.96	0.657 (0.507-0.807)	61	60	0.052
		Lactate (mmol/L)	>3.90	0.940 (0.879-0.990)	87	83	<0.001
		MSS (point)	>7.5	0.848 (0.735-0.961)	78	80	<0.001

AUC: Area under the curve, CI: Confidence interval, EP: Enzootic pneumonia, MSS: Modified respiratory scoring system, Se: Sensitivity, Sp: Specificity

Table 5. *Pearson correlation values between the modified scoring system, serum endocan, lactate, and proinflammatory cytokines in all calves (n= 80) included in the study*

Parameters		**MSS (point)**	**Endocan (ng/L)**	**Lactate (mmol/L)**	**TNF-α (ng/L)**	**IL-1β (pg/ml)**	**IL-6 (ng/L)**
MSS (point)	Pearson correlation	1	-.278*	.554**	.461**	-.228*	.262
	P		0.12	.000	.000	.042	.019
Endocan (ng/L)	Pearson correlation	-.278*	1	-.029	.106	.369**	.242*
	P	0.12		.799	.350	.001	.031
Lactate (mmol/L)	Pearson correlation	.554**	-.029	1	.546**	-.023	.461**
	P	.000	.799		.000	.843	.000
TNF-α (ng/L)	Pearson correlation	.461**	.106	.546**	1	.181	.446**
	P	.000	.350	.000		.108	.000
IL-1β (pg/mL)	Pearson correlation	-.228*	.369**	-.023	.181	1	.393**
	P	.042	.001	.843	.108		.001
IL-6 (ng/L)	Pearson correlation	.262	.242*	.461**	.446**	.393**	1
	P	.019	.031	.000	.000	.001	

** Correlation significant P<0.05, ** Correlation significant P<0.01. MSS: Modified respiratory scoring system, TNF-α: Tumor necrosis factor alfa, IL-1β: Interleukin 1 beta, IL-6: Interleukin 6*

statistically significant difference was also determined between deceased and surviving calves with EP in terms of serum endocan, TNF-α, IL-1β, and IL-6 levels. Deceased calves with EP exhibited higher serum endocan, TNF-α, IL-1β, and IL-6 levels compared to surviving calves with EP (P=0.038, P<0.001, P=0.007, P<0.001, respectively) *(Table 3)*.

In our study, in the ROC analyses performed to determine the predictive and prognostic value of serum endocan and lactate levels in calves with EP, the cut-off value of endocan for predicting EP was determined as 70.41 and lactate as 2.55. In addition, the cut-off of endocan levels for mortality prediction was determined as 64.96 and lactate as 3.90. The cut-off value of MSS for mortality prediction was 7.5 *(Table 4)*.

Pearson correlation analysis revealed a moderate, positive, and statistically significant correlation between the MSS and lactate levels (r=0.554, P<0.001). Additionally, a moderate, positive, and significant correlation was observed between MSS and TNF-α levels (r=0.461, P<0.001). Serum endocan levels showed a weak but statistically significant positive correlation with IL-1β and IL-6 (r=0.369, P=0.001; r=0.242, P=0.031, respectively). Moderate, positive, and significant correlations were also observed between lactate and TNF-α (r=0.546, P<0.001), lactate and IL-6 (r=0.461, P<0.001), as well as between TNF-α and IL-6 (r=0.446, P<0.001) *(Table 5)*.

Discussion

Respiratory tract infections are one of the most critical problems in cattle breeding that can cause serious economic losses and mortality. In addition to bacterial and viral factors, hygiene conditions, environmental factors, age, and stress factors are also reported to play a role in the occurrence of EP, which has no specific etiology. For the diagnosis of EP in calves aged 2-6 months, cough of variable severity, increased body temperature, shortness of breath, various pathologic lung sounds, and nasal discharge are usually sufficient [17]. In this study, endocan levels, which are reported to give successful results in predicting the possible prognosis in human medicine, were compared in sick calves diagnosed with EP as a result of clinical and laboratory findings and healthy calves.

Clinical scoring systems evaluate data that can be rapidly collected from patients to assess patient health and prognosis and have been used in various human and veterinary medicine applications [15,16]. For the scoring system to be applicable in field conditions: I. the patient's total score should correspond to the risk or probability of disease, II. similar scores should represent similar risks, III. objective methods should be used when scoring clinical data to optimize score performance, IV. clinical signs that are difficult to measure with sufficient precision or require expensive or time-consuming methods to measure should not be included in the scoring [16]. These data were taken into consideration in the creation of the scoring system in our study. Considering the ROC analysis (cut-off = 7.5 point, AUC = 0.848, sensitivity = 78%, specificity = 80%, P<0.001), it was concluded that it is important for veterinarians to evaluate cough (cough at least once every 10 min while the calf is at rest, 2 Point) and general condition (modarate to severe depression, 2 Point) scores in addition to low and high respiratory (<24 or >80, 2 Point) and pulse rate (<80 or >140, 2 Point) scores in determining mortality in EP.

Similar to our study, there is no information in the literature investigating endocan levels in respiratory system infections in calves. However, there are academic studies investigating endocan levels in patients with pneumonia in human medicine. Clinical studies conducted in trauma and septic patients support the hypothesis that endocan has an anti-inflammatory role, as higher endocan concentrations in the blood during admission to intensive care units appear to reduce the risk of developing ARDS. Therefore, it has been suggested that endocan secretion deficiency may be associated with a higher risk of respiratory failure and ARDS [6,18].

Gaudet et al. [19] reported that the possibility of developing ARDS in septic patients would be associated with low blood levels of endocan. Similarly, in our study, serum endocan levels were lower in calves with EP compared to healthy calves. Kechagia et al. [5] interpreted endocan as a predictive biomarker for the development of severe sepsis-induced ARDS. There is also a study indicating that endocan levels, which were significantly increased in patients with severe sepsis and septic shock compared to the control group, were lower in patients who developed acute lung injury and ARDS at 48 and 72 h compared to patients who did not [5]. The researchers explained this situation at the molecular level by decreased endocan release from pulmonary endothelial cells or increased proteolysis by neutrophil serine proteases. Gunaydin et al. [20] found lower serum endocan levels in community-acquired pneumonia patients compared to the control group (P<0.005).

Béchard et al. [12] found that endocan, a proteoglycan, binds to LFA-1 on the cell surface of leukocytes, and this complex reduces leukocyte adhesion via ICAM-1. It has been reported that the reason for lower endocan levels in relatively more severe patients may be neutrophil-derived cathepsin G, which has been shown to increase with neutrophil activation, and that this protein degrades Endocan into a 14 kDa peptide fragment [21]. The hematology device used in the study does not differentiate neutrophil, basophil, and eosinophil granulocytes. Since

neutrophil granulocytes are the most abundant granulocyte type and the most important cellular component of acute inflammation, an increase in granulocyte count was considered as an increase in neutrophil count in this study. In our study, it was determined that calves with EP had higher average GRA (10^9/L) levels than healthy calves, while dead calves had lower GRA (10^9/L) levels than surviving calves. The inverse relationship observed between endocan and GRA in our study was thought to be caused by neutrophil-derived cathepsin G protein, which was reported to increase as a result of neutrophil activation by De Freitas Caires et al. [21] and Kechagia et al. [5].

The apparently contrasting findings of lower endocan levels in calves with EP and higher levels in non-survivors may be explained by differences in the dominant pathophysiological mechanisms and disease severity. Previous studies have shown that endocan levels may decrease in conditions characterized by severe pulmonary involvement, such as ARDS and pneumonia, possibly due to reduced release from injured pulmonary endothelial cells or increased proteolytic degradation by neutrophil-derived serine proteases, particularly cathepsin G [5,19-21]. In contrast, fatal cases of sepsis are characterized by widespread systemic endothelial activation and dysfunction, leading to increased circulating endocan levels. Therefore, lower endocan levels in EP calves may reflect predominant lung-localized endothelial injury and neutrophil-mediated proteolysis, whereas higher endocan levels in non-survivors may indicate severe systemic endothelial activation associated with poor prognosis.

Lactate is produced under hypoxic conditions and poor tissue perfusion and is used as an indirect indicator of tissue hypoxia [22]. There are very few studies evaluating lactate levels in cattle with respiratory system infections [23-25]. In our study, it was determined that EP calves had higher lactate levels than healthy calves and surviving calves from the deceased calves (Cut-off value for mortality <3.90 mmol/L). Consistent with our findings, Ider et al. [25] reported that premature calves with respiratory distress syndrome (RDS) had higher lactate levels than calves in the control group. Additionally, Erdoğan et al. [26] reported in their study that calves with pneumonia and L-lactate levels above 4 mmol/L did not survive. Coghe et al. [23] and Šoltésová et al. [24] reported that plasma lactate levels increase with the severity of the disease process. This is thought to be due to an imbalance between anaerobic and aerobic metabolism and, possibly, an increasing imbalance between lactate production and lactate clearance.

TNF-α and IL-1β are powerful triggers that increase endocan synthesis in endothelial cells [11,14]. Clinically, high levels of TNF-α, IL-1β, and IL-6 are associated with severe inflammation, tissue damage, and mortality in sepsis. In neonatal sepsis studies, these cytokines are used for early diagnosis, follow-up, and prognosis determination [27]. In our study, TNF-α, IL-1β, and IL-6 levels were found to be higher in calves that died compared to those that survived. This difference may reflect variations in the stage and severity of the inflammatory response between calves with EP and those with fatal outcomes. Additionally, our study identified weak but positive and significant correlations between serum endocan levels and IL-1β and IL-6 levels. However, endocan exhibits a dual role in inflammatory conditions. While endocan expression can be increased by pro-inflammatory cytokines [11,12], circulating endocan levels have been reported to be decreased in conditions characterized by severe pulmonary endothelial damage such as pneumonia, acute lung injury, and ARDS [5,19-21]; this is likely due to impaired endothelial release or increased proteolytic degradation. Furthermore, in the present study, serum TNF-α and IL-6 levels were significantly lower in healthy calves compared with those in the EP group, whereas IL-1β levels were significantly higher in the healthy group than in calves with EP. IL-1β has been reported to play a physiological role in innate immune responses against infections, while its excessive release during pathological inflammatory processes is associated with tissue damage [28]. In addition, it has been reported that during the later stages of systemic inflammatory conditions such as sepsis, suppression of certain proinflammatory cytokines may occur [29]. Because cytokine dysregulation, activation of negative feedback mechanisms, and the predominance of anti-inflammatory responses in septic conditions may lead to attenuation of the proinflammatory cytokine response, these mechanisms may have contributed to the relative decrease in IL-1β levels observed in the present study [30]. This finding was considered to account for the lower IL-1β levels observed in calves in the EP group compared with control group.

In a study conducted by Gaudet et al. [19], it was reported that serum endocan levels showed high diagnostic accuracy in predicting ARDS, with an AUC value of 0.93 (95% CI= 0.87-1; P<0.001). In our study, based on the ROC analysis, considering the threshold value determined for serum endocan levels, it is suggested that endocan may only provide limited clinical benefit as a biomarker in the diagnosis of EP. However, when blood lactate levels were evaluated, it was found that blood lactate levels are a biomarker with superior diagnostic accuracy and clinical utility compared to endocan in predicting PE.

Tang et al. [9] reported that ARDS patients who survived had lower endocan levels compared to those who did not survive. These data are consistent with our study findings. In our study, serum endocan levels in calves with EP that survived were found to be statistically significantly lower than those in calves that died. Additionally, Behnoush et al.

[31] confirmed the high plasma endocan levels in deceased patients, which is consistent with our study findings.

Tang et al. [9] determined the AUC value to be 0.715 (95% CI = 0.555-0.875, P<0.017) when a cut-off of 4.96 ng/ml was used for plasma Endocan in predicting mortality. In the current study, however, ROC analysis revealed that serum endocan levels did not demonstrate similar success in predicting mortality. This finding indicates that endocan has limited clinical utility in the diagnosis of EP and also lacks sufficient diagnostic performance in predicting mortality. On the other hand, the prognostic value of lactate levels (>4 mmol/L) reported by Coghe et al. [23] was also supported in this study, and it was determined that mortality could be predicted with high accuracy using lactate levels in the ROC analysis. Furthermore, the ROC analysis revealed that MSS has significant discriminatory power in predicting mortality. All these findings indicate that serum endocan levels have limited clinical utility in mortality prediction, whereas blood lactate levels, consistent with the literature, continue to be a strong and reliable prognostic biomarker.

Our study has methodological limitations. The most important of these is that repeated measurements could not be performed on the calves included in the study. This limited the ability to track long-term changes in serum endocan and related proinflammatory cytokines. Another limitation of this study is that the etiological agents of EP could not be identified and bronchoalveolar lavage (BAL) sampling was not performed, limiting pathogen-specific assessment of inflammatory responses and disease severity. In addition, necropsy examinations were not conducted in deceased calves, restricting pathological–clinical correlation. Also, differences in age, sex, and breed among groups may also have influenced biomarker levels.

High endocan levels are known to have prognostic value in various inflammatory diseases. In our study, consistent with the literature, serum endocan levels were found to be lower in calves with EP compared to the control group. However, although serum endocan levels may be partially useful as a biomarker in calves with EP, it was found that they are not a successful biomarker for predicting mortality.

In conclusion, the modified scoring system was found to have significant discriminatory power in predicting mortality, while lactate levels demonstrated high diagnostic accuracy in both EP and mortality prediction. Furthermore, although endocan has been shown to have prognostic potential, this finding should be confirmed in further standardized studies involving homogeneous patient populations.

Declarations

Availability of Data and Materials: The data used in this article will be provided by the corresponding author (ET) upon request.

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Research Article

A Study on the Effect of Rutin in the Antibiotics Enhancement Controlling *E. coli* Infection in Broiler Chicken

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Abstract

This study aimed to identify synergistic antibacterial combinations containing rutin in both *in vivo* and *in vitro* settings. *Escherichia coli*, a major cause of poultry mortality, was isolated from broiler chickens and tested for antibiotic resistance using various methods, and the results showed that *E. coli* was resistant to all antibiotics, with only colistin showing a zone of inhibition. In the in vivo study, 50 one-day-old broilers were divided into five groups: control (non-infected, untreated), *E. coli* challenged (infected at 2 weeks old, untreated), rutin-treated (fed rutin at 1000 mg/kg diet without infection), *E. coli* + colistin (infected at 2 weeks old and treated with colistin at 1 g/5 L drinking water for 5 days), and rutin + colistin (fed rutin from day 1, infected at 2 weeks old, then treated with colistin). E. coli infection increased liver enzyme activity [alanine aminotransferase (ALT), aspartate aminotransferase (AST)], globulin levels, oxidative stress markers [malondialdehyde (MDA)], and tumor necrosis factor-alpha (TNF-α), while lowering total protein, albumin, and antioxidant enzyme activity. Treatment with both rutin and colistin resulted in decreased serum ALT, AST, MDA, and TNF-α, while increasing total protein, albumin, and antioxidant enzymes. In conclusion, rutin administration provided excellent protection against the adverse consequences of E. coli infection through its antibacterial, hepatoprotective, antioxidant, and anti-inflammatory properties, and the combination with colistin showed the greatest improvement, highlighting a synergistic effect.

Keywords: Antioxidant, Drug-resistant, *E. coli*, Poultry, Resistant gene, Rutin

Introduction

Avian pathogenic *Escherichia coli* are a tiny, rod-shaped bacterium that is 0.5 mm in diameter and 1-2 mm long. This gram-negative bacterium was found within the fecal flora and can lead to both systemic and local illnesses, causing significant threat to poultry businesses globally which may lead to substantial economic losses [1]. Though *E. coli* can cause extremely serious illnesses it also has a significant role in the autochthonous microbiota of humans and animals. Due to its impact on animal and human health, it is vital to require a full conclusion effective management of the disease and its fundamental needs, providing information about the epidemiological and microbiological circumstances associated with it. The possibility of pathogenic and/or resistant *E.coli* moving from animals to people through the food chain, direct contact, or contact with animal waste is quite concerning. Additionally, *E. coli* is a substantial source of resistance genes that could be the cause of human and animal treatment failures [2]. Colistin is a narrow-spectrum bactericidal antibiotic that is efficient against Gram-negative bacteria which has been applied intensively in the veterinary field primarily to avoid or control gastrointestinal infections in animals that provide food [3]. Antibiotic-resistant genes have emerged as a result of the widespread use of antibiotics, even though they have been effective in treating infectious diseases. Because of its potential to develop virulence traits, *E. coli* is becoming increasingly widespread worldwide, which worries both

people and veterinarians. These characteristics enable it to evade host defenses and withstand antibiotics [4].

A growing number of resistance genes, some acquired by horizontal gene transmission, have been discovered in *E. coli* strains in recent years. Antimicrobial resistance in *E. coli* should be taken very seriously as a public health concern since it is regarded as one of the main problems impacting both humans and animals globally [5]. As a result, the development of novel and unusual drugs to replace antibiotics has been forced by the reduced effectiveness of antibiotics brought on by resistance [6].

Due to the growing demand for food safety, natural-origin compounds like phytochemicals and plant polyphenols found in plant extracts, such as flavonoids, which have growth-promoting potential, antioxidants, and immunomodulation qualities became the new trend for usage as growth advocates and meat value enhancers and antibiotic substitutes because they are generally controlled and do not leave any harmful residue in animal products [7].

Rutin is valuable in biomedical applications because it is regarded as a safe chemical which has a number of therapeutic uses, including immune-stimulating, anti-inflammatory, anti-oxidant and anti-cancer properties. Furthermore, rutin has a significant level of reactive oxygen species scavenging ability [8]. Antibiotics are more effective against tested bacteria when combined with rutin [9].

The purpose of this study was to evaluate the protective and therapeutic potential of rutin, a natural flavonoid, against *(E. coli)* infection in broiler chickens, and to determine whether its combination with colistin produces a synergistic antibacterial effect. Specifically, the study focused on assessing both *in vitro* antibacterial activity and *in vivo* outcomes, including liver enzyme activity [alanine aminotransferase (ALT), aspartate aminotransferase (AST)], serum protein profiles, oxidative stress markers [malondialdehyde (MDA)], and inflammatory cytokine tumor necrosis factor-alpha (TNF-α). By comparing five experimental groups (control, *E. coli* challenged, rutin alone, *E. coli* + colistin, and rutin + colistin), the study aimed to clarify the extent to which rutin supplementation can mitigate the physiological and biochemical consequences of *E. coli* infection and enhance the efficacy of colistin treatment.

Material and Methods

Ethical Approval

This protocol was approved by the Animal Health Research Institute (AHRI) authorized the experimental procedure in accordance with the Agriculture Research Center (ARC) and IACUC committee in Egypt (ARC, AHRI, IACUC, 150/24).

E. coli Isolation, Culture, and Purification

Specimens from the liver, intestine, kidney, and lung were cultured on MacConkey agar, then E.M.B agar (eosin methylene blue) in an aseptic environment. The plates were tested for the distinctive *E. coli* colonies after 24 h/37°C [10].

Serological Identification of *E. Coli*

According to Kok et al[.11] the putative isolate was identified serologically using Denka Seiken Co. (Japan) as quick diagnostic *E. coli* antisera sets for the identification of the Enteropathogenic kinds, and the diagnostic sera were used to achieve slide agglutination assays.

Susceptibility Test of *E. Coli* to Antibiotics

The disc diffusion method was used to analyze the *E. Coli* strain's sensitivity pattern to twenty-two antibiotics (Oxoid, UK) in accordance with the Clinical and Laboratory Standards Institute's [12], as Doxycycline (Do, 30 µg), Erythromycin (E, 15 µg), Ciprofloxacin (CIP ,5 µg), Ampicillin (AMP, 10 µg), Streptomycin (S, 100 µg), Chloramphenicol (C, 30 µg), Amoixcillin (AX, 10 µg), Neomycin (N, 30 µg), Gentamycin (Cn, 10 µg), Colistin (CT, 10 µg), Kanamycin (K, 30 µg), Nalidixic acid (NA, 30 µg), Ampicillin/sulbactam (SAM, 20 µg), Amoxicillin/clavulanic acid (Amc, 30 µg), Sulfamethaxazole/trimethoprim (SXT, 25 µg), Levofloxacin (LEV, 5 µg), Cefotaxim (CTX, 30 µg), Tetracyclin (TE, 30 µg), Oxacillin (OX, 30 µg), Rifampin (RF, 30 µg), Fusidic acid (FA, 10 µg), Spectinomycin (SPT, 100 µg). The plates were inoculated with an *E. Coli* suspension that was adjusted to the 0.5 McFarland standard (1.5×10^8 CFU/mL), and they were cultured for 18 to 24 h at 37°C. The inhibitory zone was then measured.

The *in vitro* Antibacterial Activity of Rutin

Using the agar well diffusion technique, a well is cut using a sterilized borer (6 mm in diameter), 50 µL of rutin is dissolved in DMSO (25 g/mL), and the bacterial suspension equal to 0.5 McFarland standard ($1.5x10^8$ CFU/mL) is distributed on Mueller Hinton agar plate. The well is then incubated for 18 to 24 h at 37°C [13].

The minimal inhibitory concentration (MIC) is calculated as follows: Two-fold dilutions of rutin with concentrations of (512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL) were dispensed in tubes with a minimum capacity of 2 mL to perform a broth macro-dilution test. A microbial inoculum suspension that has been adjusted to 0.5 McFarland scale ($1.5x10^8$ CFU/mL) is then added to each tube. The infected tubes are thoroughly mixed and then incubated for 18 to 24 h at 37°C [12].

PCR Assay

Molecular identification of E. coli: Genomic DNA was extracted to serve as a template [14]. The housekeeping

gene for *E. coli* alkaline phosphatase, phoA, exists in all *E. coli* utilized to identify particularity in polymerase chain reaction (PCR) to identify maintained strains *(Table 1)*. The QIA quick PCR product extraction kit was used (Qiagen, Valencia). The sequence reaction was obtained using the PerkinElmer Bigdye Terminator V3.1 cycle sequencing kit, and purification was carried out using a Centrisep spin section. DNA sequences were obtained by A BLAST® investigation (Basic search technique for local alignment) [15] was initially conducted to generate a character sequence to accessions in GenBank using the genetic analyzer Biosystems3130 (HITACHI, Japan). The phylogenetic tree was constructed using the lasergenednastar12.1 MegAlign module [16] and analyses of Phylogenetic were prepared utilizing highest probability, maximum parsimony and neighbor-joining in MEGA6 [17].

Molecular Detection of Resistance and Virulence Genes: Real-Time PCR was used to detect genes encoding resistance to gentamicin *(aac*(3)-Ia), sulphonamides *(sul*1), trimethoprim *(dfr*A), tetracycline *(tet*A, *tet*B), colistin *(mcr*1), erythromycin *(ere*A), and aminoglycosides *(aadA*1). In addition, virulence genes including Shiga toxin *(stx*1, *stx*2) and intimin *(eae*A) were identified. Primers were provided by Metabion (Germany). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions with minor adaptations *(Table 1)*.

Table 1. *Target genes, primer sequences and amplicon sizes*

Target Gene	Primers Sequences	Product Size (Bp)
phoA	F: CGATTCTGGAAATGGC AAAAG R: CGTGATCAGCGGTGACTATGAC	720
TetA(A)	F: GGTTCACTCGAACGAC GTCA R: CTGTCCGACAAGTTGCATGA	570
TetB	F: CCTTATCATGCCAGTCT TGC R: ACTGCCGTTTTTTCGCC	773
EreA	F: GCCGGTGCTCATGAAC TTGAG R: CGACTCTATTCGATCAGAGGC	420
Aada1	F: TATCAGAGGTAGTTGG CGTCAT R: GTTCCATAGCGTTAAGGTTTCATT	484
Stx1	F: ACACTGGATGATCTCA GTGG R: CTGAATCCCCCTCCATTATG	614
Stx2	F: CCATGACAACGGACAG CAGTT R: CCTGTCAACTGAGCAGCACTTTG	779
EaeA	F: ATGCTTAGTGCTGGTTTAGG R: GCCTTCATCATTTCGCTTTC	248
Sul1	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	433
DfrA	F: TGGTAGCTATATCGAAGAATG R: GAGT TATGTTAGAGGCGAAGTCTTGGGTA	425
Mcr1	F: CGGTCAGTCCGTTTGTTC R: CTTGGTCGGTCTGTAGGG	308
aac(3)-Ia	F: TTGATCTTTTCGGTCGTGAGT R: TAAGCCGCGAGAGCGCCAACA	150

In vivo Antibacterial Activity

E coli Strain: The experimentally employed strain of *E. coli,* PP291565, was obtained from diseased birds. Each 2-week-old chicken in the infected groups received an injection of 1 mL of saline containing 10^8 *E. coli* colony forming units (CFU/mL) [18].

Rutin: Rutin is manufactured by Changsha huir Biological-tech Co., Itd China. Product name: Rutin NFI I, appearance: light yellow needle crystal powder. Part used: Whole flower bud. Extract Solvent: Ethanol & Water. Batch number: 23061514. Rutin was given to the baseline diet of chicken at a level of 1000 mg/kg from day one until the completion of the experiment [19].

Colistin: Pharma Swede, Egypt, is the manufacturer of Colistin Sulphate®. The powder dissolves in water. 5.000.000 IU of colistin sulfate are present in one gram. Administration and Dosage: Drinking water is used to administer the product orally. Poultry dosage: 1 g/5 L of drinking water for three to five days.

Experimental Design

Fifty Cobb broiler chicks (one-day old) were purchased from a commercial poultry company. Chicks were sectioned and housed in separate floor pens, each unit containing 10 chicks, under standard hygienic conditions. All chicks were vaccinated against Newcastle disease at 8 days of age and Gumboro disease at 13 days of age.

Animal Housing Conditions

Housing units: Each group of 10 chicks was housed in a separate floor pen (dimensions: 1.0 x 1.0 m).

Flooring: Pens were bedded with clean, dry wood shavings at a depth of ~5 cm, replaced weekly.

Ventilation: Natural ventilation supplemented with fans to maintain air circulation.

Temperature: Brooding temperature maintained at 32-34°C during the first week, gradually reduced by 2-3°C per week until reaching 24°C.

Humidity: Relative humidity maintained between 55-65%.

Lighting: Continuous light (23 h light:1 h dark) during the first week, then adjusted to 20 h light:4 h dark.

Stocking density: 10 chicks/m^2, in line with commercial recommendations.

Biosecurity: Pens disinfected before chick placement; footbaths and restricted access maintained throughout the experiment.

Water supply: Fresh, clean drinking water provided *ad libitum* via nipple drinkers.

Feed supply: Corn-soybean balanced ration provided *ad libitum* in trough feeders.

Group Classification

Group 1 (Negative control): Basal diet only, no challenge.

Group 2 (Positive control): Basal diet, challenged with *E. coli* at 2 weeks old.

Group 3 (Rutin group): Basal diet supplemented with rutin (1000 mg/kg feed) from day one, challenged with *E. coli.*

Group 4 (Colistin group): Basal diet, challenged with *E. coli* at 2 weeks old, then treated with colistin (1 g/5 L drinking water) for 5 days.

Group 5 (Rutin + Colistin group): Basal diet supplemented with rutin from day one, challenged with *E. coli* at 2 weeks old, then treated with colistin.

At the end of the experiment, *E. coli* was re-isolated and bacteriologically examined in all groups by obtaining samples from the colon, lung, liver, and spleen of slaughtered chickens.

Sampling

On day 21 of the experiment, blood samples were obtained from each chicken's wing vein. 3 mL of blood was drawn without an anticoagulant. The serum was isolated and stored at -20°C for biochemical analysis. Chicks were slaughtered by neck dislocation, and liver tissue samples were taken to test antioxidant levels.

Biochemical Analysis

Serum total protein determination was carried out using Spectrum &CO kit (Diuret kit) (CAT. No. 310 001). While albumin was determined using Spectrum & CO kit (CAT. No. 211 001). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were measured using spectrum kit CAT. NO. 260 001 and 265 001, respectively. While globulin was computed following [20], Malonaldehyde (MDA), reduced glutathione (GSH), and catalase (CAT) levels in liver tissue were determined with the Bio-diagnostic kit CAT.NO MD 25 29, GR 25 11, and CA 25 17, respectively. Serum tumor necrotic factor-α (TNF-α) was tested using commercially available ELISA kits, following manufacturer's instructions (R and D Systems; Minnesota; Minneapolis, USA).

Statistical Analysis

The One-Way Analysis of Variance (ANOVA) test was performed to statistically analyze the data. The Duncan test was performed after presenting the data as mean ± standard error (SE) with SPSS 14.0 (2006). The statistical significance level was $P<0.05$.

Results

E. Coli Identification

The microscopic examination exhibited gram-negative, non-sporulated, straight rods. On MacConkey *E. coli*, ferments lactose, and appears red/pink colony. While on EMB agar, colonies produced distinctive pink colonies with a metallic sheen. The serotyping of isolated *E. coli*'s discovered good results for O91:H21 latex agglutination was considered likely *E. coli* EHEC.

Antibiotic Susceptibility Test

Twenty-two antibiotic discs were applied to the *E. coli* strain, and the results showed that the *E. coli* isolate was resistant to all of the tested antibiotics, with only a 13 mm inhibition zone for colistin.

The *In vitro* Antimicrobial Activity of Rutin

By Agar Well Diffusion, Rutin showed antibacterial activity against *E. coli* PP291565 with a growth inhibition zone valued 15 mm *(Fig. 1)*. Rutin had a minimum inhibitory concentration of 128 µg/mL against the *E. coli* strain.

E. Coli Molecular Identification and Virulence and Resistance Gene Detection

The appearance of characteristic bands at 720 bp demonstrated that the 16S rRNA genes of the *E. coli* DNA samples were amplified successfully *(Fig. 2)*. The sequences, which were submitted to the Gene Bank with accession number PP291565, showed similarities to *E. coli* when compared to published sequences using the Local Alignment Search Tool. The cluster analysis and

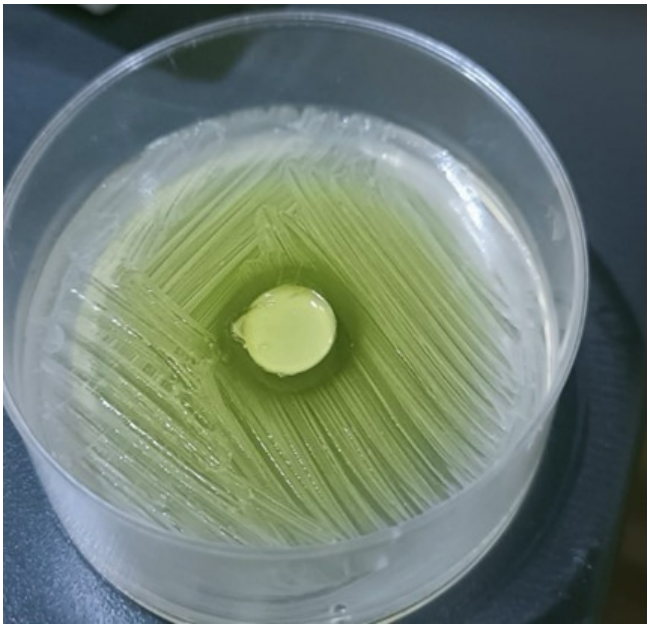

Fig 1. Antibacterial efficacy of Rutin against *E. coli* strain by agar well diffusion assay

dendrograms produced by the Local Alignment Search Tool application *(Fig. 3)*.

The existence of resistance and virulence genes is summarized in *Fig. 4, Fig. 5*, and *Fig. 6. Tet*(A) 570 bp, *Tet* (B) 773 bp, *ere*A (420 bp), *Aada*1 (484 bp), *dfr*A (425 bp) and *Mcr*1 (308 bp), *Stx*1 (614 bp), *eae*A (248 bp) and *Sul*1 (433 bp). while *aac* (3)-Ia and *stx*2 genes not detected. The isolate was classified as an enterohaemorrhagic *E. coli* (EHEC) because it possessed both the *stx* and *eae* genes.

Results of Experimental Infection

Re-isolation of *E. coli* at the end of the experimental period was negative in both the non- infected and non-treated group (control negative) (G1) and the infected group treated with Rutin and colistin together (G5). While in the infected and non-treated group (control positive) (G2), *E. coli* was isolated from all sacrificed birds. Also, it was isolated from 40% of the birds in the infected group

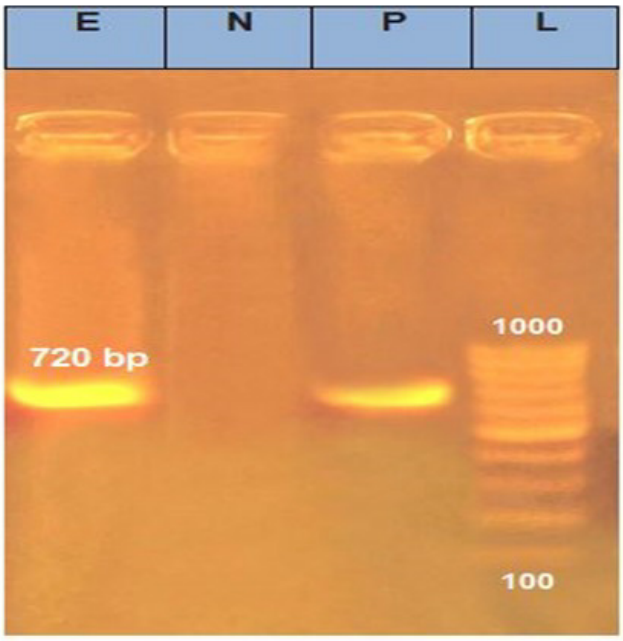

Fig 2. PCR results of the amplified 16 S rRNA gene of *E. coli*

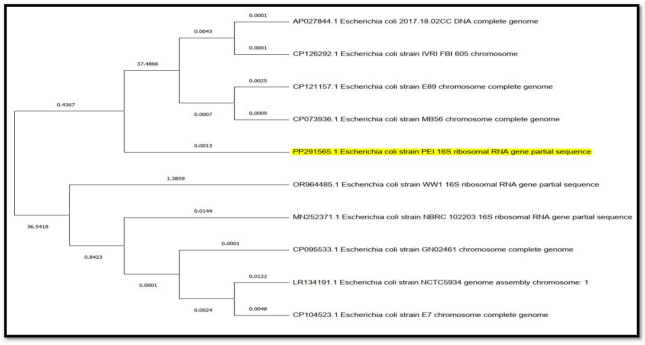

Fig 3. *Escherichia coli*'s 16S rDNA sequence similarity phylogenetic tree

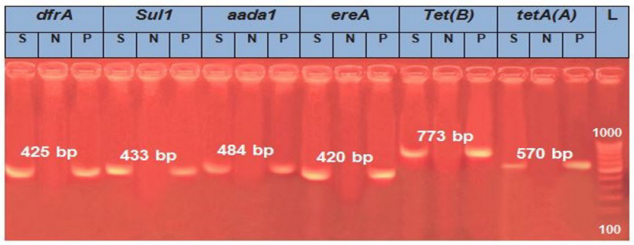

Fig 4. Gel electrophoresis of *dfrA, Sul1, aada1, ereA , Tet* (A) and *te t* (B) genes in *E. coli*

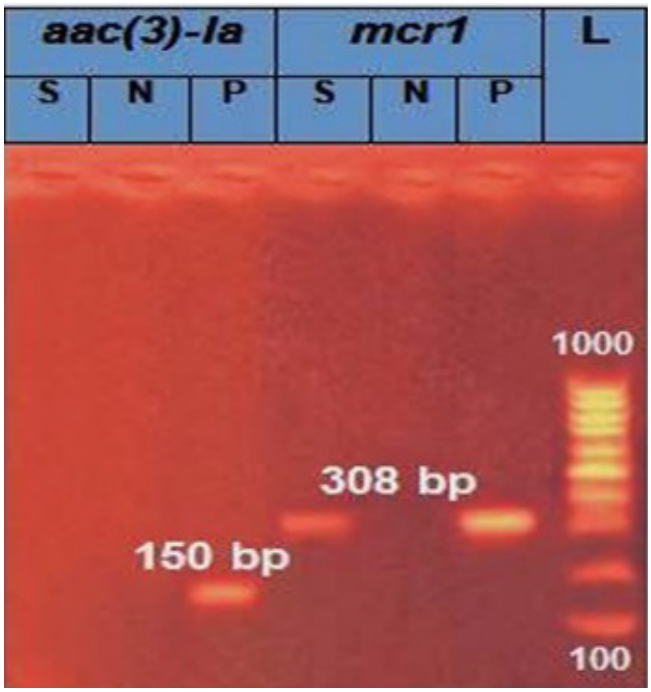

Fig 5. Gel electrophoresis of *mcr1* gene

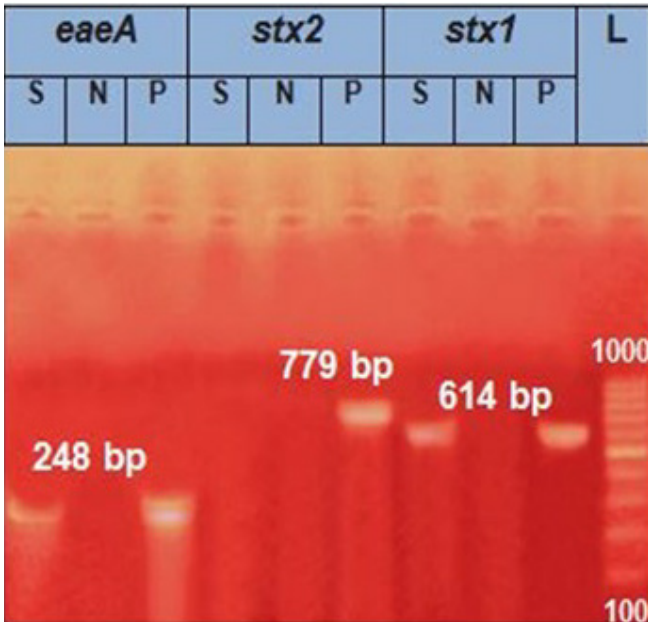

Fig 6. Gel electrophoresis of *Stx1* and *eaeA* gene

treated with Rutin only (G3). Finally in the infected group treated with colistin (G4) *E. coli* was re-isolated from 20% of the birds.

The *In vivo* Antimicrobial Activity of Rutin

The effects of rutin, colistin, and their combination on the liver functions, antioxidant enzymes, and inflammatory markers in broilers experimentally infected with E. coli were shown in *Table 2* and *Table 3.* Regarding total protein (TP) it significantly declined (P<0.05) in G2 (3.66±0.09), and G4 (3.91±0.09) in contrast to G1 (4.50±0.10), and G5 (4.37±0.11) treated with both rutin and colistin. Albumin level was significantly reduced (P<0.05) in G2 (0.81±0.03) compared to G1 (1.94±0.02), and to other groups as G3 (1.68±0.05), G4 (1.55±0.05), and G5 (1.89±0.05) *(Table 2).* Treatment with the combination of rutin and colistin in G5 revealed a non-significant change in albumin level in comparison to G1. Contrary, globulin levels were significantly increased in G2 (2.85±0.04) compared with G1 (2.56±0.02) and other groups as G3 (2.37±0.03), G4 (2.35±0.04) andG5 (2.48±0.05). The liver function parameters, as presented in *Table 2,* showed that AST activity of G2 had significantly (P<0.05) elevated activity reaching (92.03±2.09) compared to G1 group (66.70±1.19). Rutin and colistin treatment caused significant improvement in the AST activity in comparison to the infected control group reaching (77.33±1.20) and (79.03±2.07) in G3 and G4 respectively. Likely, G5 the infected and treated group with a combination of rutin and colistin had normal AST activities (68.33±2.03) compared to G1. Moreover, G1 had ALT activity of (16.17±1.01), while G2 had significantly (P<0.05) higher ALT activity of (29.84±1.00). Such values within G3 and G4 were significantly reduced to (21.03±0.58) and (22.63 ±0.69). However, G5 did not have essentially different ALT activity reaching the normal levels of (18.02±0.58).

Regarding the antioxidant status, the obtained findings in *Table 3* showed that CAT activity of (G1) was (102.40±2.75), while G2 had significantly (P<0.05) lower CAT activity of (56.17±2.17). Such values in G3 and G4 were significantly increased reaching (88.33±2.04) and (79.03±1.79) compared to G5. G5 did not have significantly different CAT activity than G1 reaching (96.69±1.35).

Regarding MDA concentration, G2 had significantly (P<0.05) elevated levels reaching (12.60±1.06) compared to G1 (4.35±0.18). Rutin caused significant improvement in the MDA concentration reaching levels of (5.43±0.19) in G3. Likely, G4 and G5 the infected and treated groups with colistin alone or in combination with rutin had relatively reduced MDA concentrations of (6.59±0.02) and (5.13±0.16), respectively in comparison to the infected non-treated group.

Referred to GSH activity, it was shown that after treatment, G5 had the highest activity (28.06±0.24), followed by G3 (26.69±0.27), G4 (24.65±0.50), while the infected non-treated group had the least GSH activityG2 (16.06±1.04) compared with G1(29.45±0.57)

Regarding the inflammatory marker, TNF-α, it was much upregulated (P<0.05) in G2 (385.03±6.14) compared to G1 (261.66±2.95), and to other groups as G3 (292.66±3.18), G4 (293.33±2.40), and G5 (279.21±1.42) *(Table 3).*

Table 2. *The impact of colistin, rutin, and their combination on the liver function of broilers subjected to experimental E. coli infection*

Parameters	Groups				
	G1	G2	G3	G4	G5
Total protein (g/dL)	4.50±0.10 ^{a}	3.66± 0.09 ^{d}	4.04±0.10 ^{bc}	3.91±0.09 ^{c}	4.37±0.11 ^{ab}
Albumin (g/dL)	1.94±0.02 ^{a}	0.81±0.03 ^{d}	1.68±0.05 ^{b}	1.55±0.05 ^{b}	1.89±0.05 ^{a}
Globulin (g/dL)	2.56±0.0 2 ^{b}	2.85±0.04 ^{a}	2.37±0.03 ^{c}	2.35±0.04 ^{c}	2.48±0.05 ^{bc}
AST (U/L)	66.7±1.19 ^{c}	92.03±2.09 ^{a}	77.33±1.20 ^{b}	79.03±2.07 ^{b}	68.33±2.03 ^{c}
ALT (U/L)	16.17±1.01 ^{c}	29.84±1.0 ^{a}	21.03±0.58 ^{b}	22.63±0.69 ^{b}	18.03±0.58 ^{c}

Table 3. *The impact of colistin, rutin, and their combination on inflammatory and antioxidant indicators in broilers experimentally infected with E. coli*

Parameters	Groups				
	G1	G2	G3	G4	G5
CAT (U/g liver tissue)	102.40±2.75 ^{a}	56.17±2.17 ^{d}	88.33±2.04 ^{b}	79.03±1.79 ^{c}	96.70±1.35 ^{a}
MDA (nmol/g liver tissue)	4.35±0.18 ^{d}	12.60±1.06 ^{a}	5.43±0.19 ^{c}	6.59±0.02 ^{b}	5.13±0.16 ^{c}
GSH (nmol/g liver tissue)	29.45±0.57 ^{a}	16.06±1.04 ^{d}	26.69±0.27 ^{b}	24.65±0.50 ^{c}	28.06±0.24 ^{a}
TNF (Pg/mL)	261.67±2.95 ^{d}	385.03±6.14 ^{a}	292.67±3.18 ^{b}	293.33±2.40 ^{b}	279.22±1.42 ^{c}

Data are expressed as mean ± SE
a,b,c,d *Superscript: Mean significance difference among groups on P<0.05*

Discussion

The necessity to generate a novel antibacterial drug in the period of antibiotic resistance has spurred researchers worldwide to revert to natural medicine techniques to explore the potential antibacterial mechanisms of action of compounds originating from plants, e.g. polyphenols, particularly rutin [21].

In this work, *E .coli* isolated from the broiler was investigated for the presence of virulence and antibiotic resistance genes, this is a public health concern due to the frequent interactions between people and companion animals.

Multiple tetracycline determinants were existent in *E. coli* PP291565 isolated strain in agreement with Van et al.[22] studies that stated the presence of *tet*A and *tet*B genes.

Resistance to streptomycin and gentamicin is mostly dependent on the *aa*d (A1) and *aac3* genes respectively which was investigated by Szczepanowski et al.[23]. In our study we detected only *aa*d (A1) gene while *aac3* was negative by PCR assay.

Our results agrees with Shahrani et al.[24] and other studies who detected the presence of *stx* gene encodings shiga toxin which reflects a major virulent component in the pathogenicity of the EHEC pathotypes. Likewise our study revealed that *E. coli* isolate was containing genes that code Shiga toxin *(stx1)* and intimin production *(eae),* which are essential virulence determinants in *E. coli* connected with human infection but he also detected *sxt2* gene which was not detected in our study.

Poirel et al.[25] stated that the mcr-1 gene, which encodes colistin resistance, has predominantly been discovered in environmental and animal samples since its discovery worldwide, as well as in *E. coli* isolated from broiler samples.

Due to the existence of poultry products that contain antibiotic residues, and because of growing user demand for products free from antibiotic residues, have accelerated the look for options that might exchange antibiotics that do not reduce production or product value.

Our result revealed that Rutin is one of the antibiotic alternates for *E. coli* with MIC value of 128 µg/mL that agrees with Miklasińska-Majdanik et al.[26] who reported that RH demonstrated variable effects against *E. coli*.

Several studies have revealed that natural-origin compounds have activity against bacteria alone and in combination with specific antibiotics [7]. In our study, we have concentrated on the antibacterial impact of Colistin and Rutin against *E. coli* which could have useful uses in the era of resistance to antibiotics. Also that agrees with Wang et al.[27] who stated that rutin has antibacterial activity against both Gram-positive and Gram-negative bacterial strains.

Serum globulin concentration, AST, and ALT activity were significantly higher in the *E. coli*-infected non-treated group as *E. coli* infection has a characteristic symptom that damages the liver in chicken is perihepatitis [28]. Similar results were observed previously by Ghandour et al [29]. The elevated globulin levels may result from the birds' immune response to *E. coli* infection. The findings aligned with the previous findings [30]. Elevated serum AST signifies cellular damage to cardiac myocytes and hepatocytes, while increased serum ALT is predominantly due to hepatic diseases. These findings are consistent with the studies conducted by Abd-Allah et al.[31]. That Increase in hepatic enzyme activity may be associated with modified hepatocyte membrane permeability induced by the microorganism; consequently, the cell membrane's functional integrity is compromised, leading to the efflux of these enzymes into the bloodstream [30]. Bacterial toxins impact liver cells, especially those next to the central vein, resulting in an increase in ALT activity. These cells get the least amount of nutrients from the blood and are very vulnerable to hepatotoxins and inflammatory chemicals, which directly affect how permeable the membranes of liver cells are [32].

According to the current study, dietary rutin supplementation significantly affects serum AIT levels. This suggests that rutin has hepato-protective qualities, which may be related to the production of cytokines, which have been shown to provide hepato-protection in a variety of liver injury models [33].

Biochemical assays showed a significant decrease in albumin which is the carrier to anions, cations, fatty acids, and hormones. Changing the rate at which they are made affects the amounts of these compounds and the physiological responses that birds need to stay alive [34]. Declined result of total protein concentration, in agreement with Vikash et al.[35], who posted that this hypoproteinemia may be associated with renal pathology, leading to protein depletion, hepatic impairment resulting in impaired plasma protein synthesis, or congestive heart failure. Hyperglobulinemia in *E. coli* infection has been documented by Abd El-Ghany et al.[36], associated with liver cirrhosis, hepatitis, and Kupffer cell expansion.

The *E. coli*-infected non-treated group's CAT and GSH activity was significantly lower than that of the control group, according to the data regarding antioxidant status. Indicators of oxidative stress and inflammation, malondialdehyde (MDA) levels were significantly greater ($P<0.05$) in the infected non-treated group than in the negative control group. The findings are consistent with those of El-Kilany et al.[37] who found that broiler SOD and GPX levels were lowered as a result of reactive oxygen species build up and oxidant/antioxidant imbalance brought on by an *E. coli* infection. Furthermore,

hens infected with *E.coli* had considerably decreased antioxidant enzyme levels and higher MDA [29]. These alterations may result from the bacterial LPS (endotoxin) it produces damages multiple organs, including the liver, by boosting lipid peroxidation and generating reactive oxygen intermediates [38].

Natural antioxidants, such as flavonoids, taking a great concern for preserving health and preventing disease. Flavonoids have been shown to be superior antioxidants that can enhance T-SOD and CAT activity while lowering MDA levels, which is consistent with our current findings [8]. As a powerful antioxidant flavonoid, rutin exhibits its antioxidant properties by chelating iron to eliminate free radicals, hence preventing lipid peroxidation and xanthine oxidase activity [39].

According to Tufarelli et al.[40], rutin supplementation significantly increased GSH and CAT activities while decreasing malonaldehyde levels in the current study. This suggests that rutin has the ability to transfer electrons and free radicals in addition to its capacity to activate antioxidant enzymes and lessen oxidative stress Rutin's chemical makeup may directly scavenge ROS, according to the first reports of its antioxidant qualities *in vitro* and *in vivo* [41]. Second, it increases GSH production, and it is believed that enhanced expression of different antioxidant enzymes, including SOD and CAT, promotes cellular oxidative defense mechanisms [42].

Tumor necrosis factor TNF-α is an inflammatory cytokine, which can cause hepatocytes to generate a number of acute-phase proteins during the acute phase response. Broilers infected with *E. coli* most likely had higher levels of cytokines and inflammatory markers, including TNFα. The idea that infection-induced tissue damage requires increased production of inflammatory cytokines is further supported by the current study's findings regarding elevated tissue levels of the pro-inflammatory cytokines TNF-α following *E. coli* tumor necrosis factor (TNF) [43]. TNFα was considerably ($P<0.05$) higher in the experimentally infected group than in the treated and negative control groups [29].

Numerous studies have been conducted to comprehend the pharmacological processes and efficacy of flavonoids, including rutin, since they have been identified as naturally occurring anti-inflammatory compounds a great deal of research has been done to understand their pharmacological mechanisms and effectiveness [44]. In line with our findings, rutin has potent anti-inflammatory properties by lowering levels of pro-inflammatory molecules such TNF-a, and NF-κB production [45]. Our results collectively imply that rutin may enhance immune response and reduce inflammation in broilers by blocking the NF-κB signaling pathway.

The effectiveness of colistin is impacted by the widespread use of antibiotics to treat germs that are resistant to many drugs, especially Enterobacteriaceae. Additionally, as was primarily shown in a number of *E. coli* strains, it was contributing to the development of colistin-resistant bacteria [46]. On the other hand, the limited gastrointestinal absorption of colistin, even in contaminated animals, indicates that oral colistin administration is increasing colistin resistance by placing selection pressure on the intestinal flora of animals due to antibiotics [47]. Numerous researches have demonstrated that using certain flavonoids reduces antibiotic resistance and reveals vulnerability in a synergistic manner, providing drug-resistant bacteria with a significant therapeutic strategy [48].

Food safety demands that plant extracts and their phytochemicals (such as flavonoids) to become the new trend for use as growth promoters and meat quality improvers. In the current study, rutin significantly improved antioxidant status activity more than colistin therapy, bringing it back to normal levels in group 5 treated with a combination of rutin and colistin. It is suggested that resuming the antibacterial activities of antibiotics when flavonoids are present may be caused by flavonoids' ability to improve the efficiency of the antibiotic medicine [49].

From this study we conclude that the most serious issue in this research is that the demonstrated *E. coli* PP291565 (EHEC) isolate not only was resistant to 95% of the antibiotics tested but also its molecular screening clarified that it carries multiple resistance and virulence genes, which makes it very virulent, difficult to be treated. But rutin demonstrated a considerable growth inhibition zone and MIC value against *E. coli in vitro,* and a synergistic spectacular impact with colistin *in vivo.*

In conclusion, rutin administration provided excellent protection against the adverse consequences of *E. coli* infection through its anti-bacterial, hepato-protective, antioxidant, and anti-inflammatory properties. With the colistin treatment combination, it shows the greatest improvement. So that the synergistic effect of rutin with colistin suggests that rutin can convert antimicrobial resistance pathways into susceptible ones, restoring the drug's efficacy which raised hopes that it could be used as a future treatment for highly resistant and virulent *E. coli.*

Declarations

Availability of Data and Materials: The data sets analyzed during the current study are available from the corresponding author S. M. Elmesalamy on reasonable request.

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RESEARCH ARTICLE

Effects of Intranasal Butorphanol-Diazepam Combination on Ultrasonographic Ocular Biometry, Tear Production, and Intraocular Pressure in Yellow-Legged Gulls (*Larus michahellis*)

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Abstract

The aim of this study was to evaluate the effects of intranasal butorphanol-diazepam sedation on tear production, intraocular pressure (IOP), and ocular biometry in Yellow-legged Gulls (*Larus michahellis*). Eighteen gulls (nine juvenile, nine adult) undergoing rehabilitation were included. Measurements from the left (n=18) and right (n=18) eyes were analyzed separately for Schirmer Tear Test I (STT-I), IOP, and ultrasonographic parameters. Sedation was achieved with intranasal diazepam (8mg/kg) and butorphanol (1mg/kg). The body weights of the gulls were 670.55±35.84 g for juveniles and 782.22±61.61 g for adults. The sedation protocol used in this study produced statistically significant decreases (P<0.05) in juvenile animals in STT-I, anterior chamber depth (ACD), vitreous chamber depth (VCD), axial globe length (AGL), equatorial globe width (EGW) and pecten oculi length (POL). Statistically significant increases (P<0.05) were detected in lens axial height (LAH) and axial globe height (AGH). In adults, statistically significant decreases (P<0.05) were observed in central corneal thickness (CCT), ACD, and VCD while LAH, EGW and AGH showed statistically significant increases (P<0.05). Although decreases in IOP and increases in lens thickness (LT) were noted in both groups, no statistically significant differences were detected. These results indicate that intranasal butorphanol-diazepam provides reliable sedation for ophthalmic assessment in Yellow-legged Gulls without adverse effects; however, its influence on ocular parameters should be considered when interpreting measurements under sedation.

Keywords: Gulls, Intraocular pressure, Sedation, Schirmer test, Ultrasound

INTRODUCTION

The Yellow-legged Gull (*Larus michahellis*; family Laridae) is a common coastal seabird along the shores of Samsun, Türkiye. It is omnivorous, feeding on fish, crustaceans, worms, and the eggs and chicks of other birds [1]. Adults are characterised by long wings, a broad hooked bill with a prominent red spot extending onto the upper mandible, and a darker dorsum with a bluish hue. Although populations have increased markedly in recent decades and the species is considered invasive, information on its visual physiology and ocular anatomy remains limited [1].

Handling and restraint of wild birds often provoke pronounced stress responses. Sympathetic stimulation and catecholamine release may lead to tachycardia, tachypnoea, hyperthermia, and hypertension, with sudden death possible in compromised individuals [2,3]. Sedation reduces responsiveness to external stimuli, suppresses struggling and vocalisation, and minimises the risk of injury to handlers and stress-related complications in patients. This is particularly valuable in aggressive species such as gulls and raptors, facilitating examination, diagnostic imaging, sample collection, and minor procedures [2]. Intranasal administration offers a practical, non-invasive route for sedation, especially in field settings where intravenous access may be challenging; however, its effects on ocular parameters in this species have not been documented.

Species-specific ophthalmic reference values are essential for diagnosis and clinical decision-making. Tear production, which reflects corneal epithelial health, is commonly assessed using the Schirmer tear test I (STT-I), although modifications exist [4,5]. Measurement of intraocular pressure (IOP) is critical for identifying ocular hypertension and the risk of glaucoma, as sustained elevation can cause irreversible retinal and optic nerve damage [4,6]. Ocular ultrasonography is a routine, non-invasive modality for assessing ocular structures based on echogenic differences [7].

The objective of this study was to defines baseline values for tear production, IOP, and ocular ultrasonography in Yellow-legged Gulls, and to determine whether intranasal butorphanol-diazepam sedation influences these parameters. Given the significant interspecies variation in avian ocular anatomy, morphometric values obtained from one species cannot be reliably applied to another without risking diagnostic misinterpretation. Furthermore, recognising potential differences between sedated and non-sedated measurements is crucial for accurate clinical assessment. Accordingly, species-specific and sedation-dependent reference values provide a necessary foundation for distinguishing physiological variation from pathological change and thereby support precise clinical diagnosis and appropriate management in avian ophthalmology [4,8].

Material and Methods

Ethical Statement

All procedures were approved by the Ondokuz Mayıs University Animal Experiments and Local Ethics Committee (Approval No: 2025-11). This study was conducted with the permission of the Republic of Türkiye Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (Ref. No: E-72784983-288.04-18471322, Date: 17.03.2025).

Animals

The study population comprised Yellow-legged Gulls *(Larus michahellis)* that had completed treatment and rehabilitation at the Ondokuz Mayıs University. The gulls were housed in a 1 m^3 (1 x 1 x 1 m) stainless steel cage in a room with a 22-24°C temperature and 40%-60% humidity, with a 12 h light and 12 h dark cycle. All patients were provided with ad libitum water and 100 g of fresh fish twice daily. All birds were capable of oral food intake, demonstrated flight ability, and were scheduled for imminent release into the wild. Sex could not be determined, and none presented with ocular pathology. A total of 18 gulls (nine juveniles and nine adults; 36 eyes) were examined. Age determination of the specimens in this study was performed based on plumage characteristics, bill and iris coloration, following the standard criteria previously established for the Yellow-legged Gull. Adult individuals (≥4 years old) were defined as those exhibiting a fully white head, pale grey mantle, characteristically patterned black-and-white wing tips, yellow bill with a red gonys spot, pale iris, and fully developed plumage. Juvenile individuals were characterised by pink legs; a dark bill; a brown iris; a grey-streaked head; grey-brown feathers with pale edges on the upperparts and wing coverts, underparts mottled grey-brown; primaries and secondaries dark, with similar pattern; primary coverts dark brown; white tail with broad dark terminal band. This classification ensured clear and reliable comparative analyses between age groups [9,10].

Experimental Design and Procedure

Ophthalmic examinations were performed between 09:00 and 16:00 h. Initial examinations were performed without sedation. Assessments were conducted in the following order: Schirmer tear test I (STT-I), intraocular pressure (IOP) measurement, and ocular ultrasonography. The left eye was examined first, followed by the right. Ultrasonography was performed last, as acoustic gel and probe application could potentially influence tear production and IOP. Sedated examinations were repeated 24 h later using a neuroleptanalgesic protocol. After a 4-hour fasting period, each gull underwent the sedation protocol.

Drugs and Intranasal Administration

Diazepam 8 mg/kg (Diapam 10 mg/2 mL I.M./I.V, Osel Pharmaceuticals®, Türkiye) and butorphanol 1 mg/kg (Nalgosed 10 mg/mL, Bioveta Pharmaceuticals®, Türkiye) were combined in a single syringe and administered intranasally via a 22 G intravenous catheter with the needle removed. The catheter was inserted approximately 5-6 mm into each nostril, and the solution delivered slowly and evenly between both nares *(Fig. 1)*. Deep sedation was defined as the absence or marked reduction of reflexes such as wing extension/withdrawal and pedal withdrawal. The same examination protocol was then repeated.

Fig 1. Intranasal administration of butorphanol-diazepam combination

Ophthalmic Examination

Tear production was measured using STT-I strips placed in the conjunctival fornix for 1 min *(Fig. 2)*. IOP was assessed using a rebound tonometer (TonoVet®, Icare Finland Oy, Vantaa, Finland) applied perpendicularly to the corneal surface *(Fig. 3)*. Each IOP measurement was repeated three times, and mean values recorded. Ocular ultrasonography was performed with a Vetus 9 ultrasound system (Mindray Animal Care, Shenzhen, China) equipped with a C11-3s convex array transducer *(Fig. 4)*. With the probe oriented horizontally, the following parameters were measured: central corneal thickness (CCT), anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD), axial globe length (AGL), equatorial globe width (EGW), and pecten oculi length (POL). With the probe held vertically, lens axial height (LAH) and axial globe height (AGH) were recorded *(Fig. 5)*.

Statistical Analysis

Data normality was assessed using the Kolmogorov-Smirnov test. Descriptive statistics were presented as mean ± standard deviation (SD). To ensure data homogeneity and address potential age-related physiological variations, the study population was stratified into two groups: Juveniles and Adults. Comparisons between non-sedated (baseline) and sedated measurements for ocular parameters (IOP, STT-I, and ultrasonographic indices) were performed within each age group using the Paired Samples t-test. Comparisons of baseline values between the juvenile and adult groups were conducted using the Independent Samples t-test. A P-value of <0.05 was considered statistically significant. All analyses were

Fig 2. Measurement of tear production using STT-I

Fig 3. Measurement of intraocular pressure using a rebound tonometer

Fig 4. Ocular ultrasonography

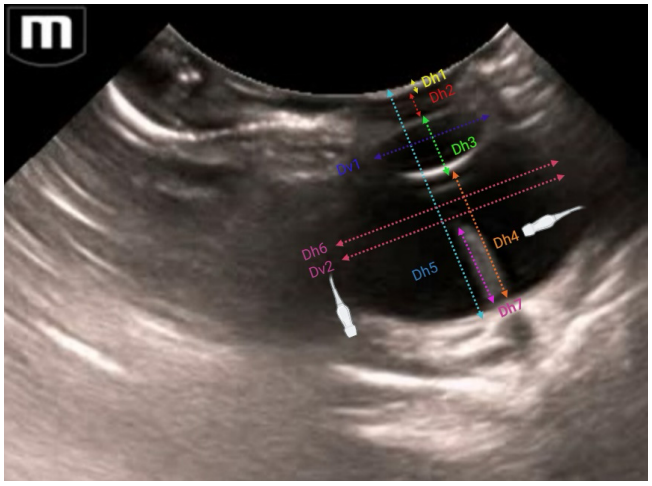

Fig 5. With the probe held horizontally, central corneal thickness (Dh1), anterior chamber depth (Dh2), lens thickness (Dh3), vitreous body depth (Dh4), axial globe length (Dh5), eyeball centre width (Dh6) and pecten oculi length (Dh7) were measured. When the probe was held vertically, lens height (Dv1) and eyeball centre height (Dv2) were measured

conducted using SPSS software (version 21.0; IBM Corp., Armonk, NY, USA).

Results

The body weights of the Yellow-legged Gulls were recorded as 670.55±35.84 g for juveniles and 782.22±61.61 g for adults. During examinations without sedation, gulls exhibited continuous wing flapping, resistance, and pecking behaviour. In particular, during ocular ultrasonography and the Schirmer I test, birds continued to struggle despite gentle restraint of the beak by assisting personnel, making examinations difficult to perform. In contrast, all examinations in sedated gulls were completed smoothly and without complication. The protocol provided an adequate depth of sedation for ocular ultrasonography and other ophthalmic assessments in all individuals. The mean onset time of deep sedation was 11.7±3.4 min.

No statistically significant differences were detected between left and right eyes (P>0.05); however, measurements from each eye were analyzed separately for STT-I, IOP, and ultrasonographic parameters. When the same parameters under sedated and non-sedated conditions were compared between juvenile and adult groups, no statistically significant differences were detected (P>0.05) *(Table 1)*. In the analysis, measurements were compared within each age group (juvenile and adult) for sedated and non-sedated parameters. This approach ensured the homogeneity of comparisons within each group, providing a fair and balanced evaluation. Accordingly, *Table 2* presents the parameters for juvenile and adult gulls, with sedated and non-sedated values shown separately within each age group.

The sedation protocol produced statistically significant decreases (P<0.05) in juveniles regarding STT-I, ACD,

Table 1. *Comparison of non-sedated and sedated ocular measurements between juvenile and adult gulls*

Ocular Parameters	Group	n (Eye)	Mean ± SD	P-value
IOP (mm/Hg)	J	18	12.278±1.127	0.920
	A	18	12.222±2.045	
IOPSED (mm/Hg)	J	18	9.388±1.5771	0.075
	A	18	10.888±3.085	
STT-I (mm)	J	18	12.055±3.171	0.104
	A	18	13.667±2.589	
STT-ISED (mm)	J	18	10.166±3.776	0.801
	A	18	9.889±2.698	
CCT (cm)	J	18	0.059±0.007	0.833
	A	18	0.060±0.008	
CCTSED (cm)	J	18	0.060±0.006	0.380
	A	18	0.058±0.008	
ACD (cm)	J	18	0.151±0.023	0.475
	A	18	0.157±0.025	
ACDSED (cm)	J	18	0.144±0.029	0.629
	A	18	0.149±0.025	
LT (cm)	J	18	0.452±0.021	0.180
	A	18	0.462±0.022	
LTSED (cm)	J	18	0.466±0.033	0.319
	A	18	0.475±0.021	
LAH (cm)	J	18	0.875±0.045	0.101
	A	18	0.899±0.040	
LAHSED (cm)	J	18	0.893±0.045	0.291
	A	18	0.911±0.057	
VCD (cm)	J	18	0.999±0.057	0.540
	A	18	1.008±0.026	
VCDSED (cm)	J	18	0.990±0.047	0.401
	A	18	1.003±0.047	
AGL (cm)	J	18	1.672±0.076	0.237
	A	18	1.704±0.082	
AGLSED (cm)	J	18	1.656±0.058	0.135
	A	18	1.688±0.066	
EGW (cm)	J	18	1.753±0.051	0.605
	A	18	1.742±0.075	
EGWSED (cm)	J	18	1.746±0.077	0.838
	A	18	1.751±0.085	
AGH (cm)	J	18	1.779±0.075	0.454
	A	18	1.761±0.061	
AGHSED (cm)	J	18	1.788±0.080	0.283
	A	18	1.760±0.076	

Table 1. Continue

Ocular Parameters	Group	n (Eye)	Mean ± SD	P-value
POL (cm)	J	18	0.680±0.022	0.054
	A	18	0.663±0.028	
POLSED (cm)	J	18	0.671±0.025	0.541
	A	18	0.677±0.034	

n: *total samples,* ***SED:*** *sedated,* ***A:*** *adult,* ***J:*** *juvenile,* ***CCT:*** *central corneal thickness,* ***ACD:*** *anterior chamber depth,* ***LT:*** *lens thickness,* ***VCD:*** *vitreous chamber depth,* ***AGL:*** *axial globe length,* ***EGW:*** *equatorial globe width,* ***POL:*** *pecten oculi length,* ***LAH:*** *lens axial height,* ***AGH:*** *axial globe height*

Table 2. *Non-sedated and sedated ocular measurements in juvenile and adult gulls*

Ocular Parameters	Juvenile (n=18 eye)		Adult (n=18 eye)	
	Mean ± SD	*P-value*	Mean ± SD	*P-value*
IOP (mmHg)	12.278±1.127	0.507	12.222±2.045	0.208
IOPSED (mmHg)	9.388±1.577		10.888±3.085	
STT-I (mm)	12.055±3.171	0.007*	13.667±2.589	0.127
STT-ISED (mm)	10.166±3.776		9.889±2.698	
CCT (cm)	0.059±0.007	0.345	0.060±0.008	0.001*
CCT SED (cm)	0.060±0.006		0.058±0.008	
ACD (cm)	0.151±0.023	0.005*	0.157±0.025	0.001*
ACDSED (cm)	0.144±0.029		0.149±0.025	
LT (cm)	0.452±0.021	0.764	0.462±0.022	0.082
LTSED (cm)	0.466±0.033		0.475±0.021	
LAH (cm)	0.875±0.045	0.001*	0.899±0.040	0.001*
LAHSED (cm)	0.893±0.045		0.911±0.057	
VCD (cm)	0.999±0.057	0.001*	1.008±0.026	0.001*
VCDSED (cm)	0.990±0.047		1.003±0.047	
AGL (cm)	1.672±0.076	0.001*	1.704±0.082	0.192
AGLSED (cm)	1.656±0.058		1.688±0.066	
EGW (cm)	1.753±0.051	0.006*	1.742±0.075	0.001*
EGWSED (cm)	1.746±0.077		1.751±0.085	
AGH (cm)	1.779±0.075	0.001*	1.761±0.061	0.006*
AGHSED (cm)	1.788±0.080		1.760±0.076	
POL (cm)	0.680±0.022	0.002*	0.663±0.028	0.340
POLSED (cm)	0.671±0.025		0.677±0.034	

** P<0.05, parameters showing statistically significant differences*
n: *total samples,* ***SED:*** *sedated,* ***CCT:*** *central corneal thickness,* ***ACD:*** *anterior chamber depth,* ***LT:*** *lens thickness,* ***VCD:*** *vitreous chamber depth,* ***AGL:*** *axial globe length,* ***EGW:*** *equatorial globe width,* ***POL:*** *pecten oculi length,* ***LAH:*** *lens axial height,* ***AGH:*** *axial globe height*

VCD, AGL, EGW, and POL, while statistically significant increases (P<0.05) were detected in LAH and AGH. In adults, statistically significant decreases (P<0.05) were observed in CCT, ACD, VCD, and AGH, whereas LAH and EGW showed significant increases (P<0.05). Although decreases in IOP and increases in LT were noted in both groups, no statistically significant differences were detected.

On ultrasonography, the cornea appeared hyperechoic with a slightly convex contour in all gulls. The anterior chamber was anechoic, consistent with its aqueous humour content. The lens was clearly delineated, with both anterior and posterior capsules visible as hyperechoic lines. The vitreous body was anechoic, and the pecten oculi was visualised at the 5 o'clock position as a tubular echogenic structure extending from the retina. The retina appeared as a hyperechoic line, separated from the orbital wall by a thin anechoic interface. The scleral and choroidal layers could not be differentiated, and equatorial and axial globe dimensions could therefore not be measured with precise margins. In these regions, measurements were taken approximately by aligning the probe parallel to the lens and relative to the ciliary region *(Fig. 5)*. The shape of the globe was conical in all birds.

Discussion

Establishing normal anatomical and physiological values for the eye in healthy wild birds is essential for diagnosing ocular pathologies that may arise in clinical or rehabilitation settings. Ophthalmological studies in wild and exotic avian species have demonstrated that ocular parameters can vary markedly between species, and in some cases, even between breeds within a species [4,11,12]. Several investigations have reported normal ocular values for a variety of bird species [13]. By focusing on a single species, our study presents STT-I, IOP, and ultrasonographic ocular biometry values in clinically healthy, rehabilitated *L. michahellis* suitable for release into the wild.

Several methods have been employed to evaluate tear production in birds, including Schirmer I, modified Schirmer, and phenol red thread tests [14]. The choice of test may vary according to the size and anatomical configuration of the conjunctival fornix. As each method reflects different layers of the tear film, they should not be compared directly [14, 15]. Accordingly, when establishing reference values, the specific test used must be explicitly reported. STT-I values are also thought to vary with orbital size and the relative dimensions of the lacrimal glands [14]. In our study, although STT-I values decreased with sedation in both juvenile and adult animals, this reduction was not statistically significant. When the groups were compared within themselves, allowing for a more homogeneous evaluation, the decrease in STT-I values remained notable, and this reduction was statistically significant in juveniles (P=0.007). This finding is consistent with the well-recognised transient xerophthalmic effect of sedatives and opioids [16]. Similar reductions in STT-I values following sedation have been reported in other species with

different sedative and anaesthetic protocols [16-18]. In wild and exotic birds, IOP has been measured using indentation (Schiøtz), applanation (Tono-Pen), and rebound (TonoVet) tonometers [19,20]. IOP values have been shown to vary across parrot and raptor species, as well as depending on the device used for measurement [19-21]. In our study, TonoVet rebound tonometry revealed no significant differences in IOP between juvenile and adult groups. Similarly, when age groups were evaluated within themselves, sedation caused a noticeable decrease in IOP values; however, this reduction was not statistically significant (P>0.05). Anaesthetic agents and clinical procedures are known to exert variable effects on IOP. Ketamine, for instance, increases extraocular muscle tone and may raise IOP, whereas most other sedatives and anaesthetics generally reduce IOP depending on the depth of anaesthesia [22,23]. Nevertheless, some studies have reported minimal changes in IOP with ketamine anaesthesia [22]. IOP is also influenced by systemic blood pressure, head and neck positioning, and body posture [24]. Therefore, it is important to characterise species-specific effects of sedatives and anaesthetics on IOP.

When juveniles and adults were compared with each other -both for non-sedated and sedated measurements- no significant differences were detected for any ultrasonographic parameter (P>0.05). However, when each age group was evaluated within itself, certain changes became apparent. In both juveniles and adults, a decrease in IOP (P=0.507) and an increase in LT (P=0.208) were observed, but these changes were not statistically significant. For all remaining parameters, significant changes were detected either within the juvenile group, the adult group, or within both age groups. While Lens Thickness (LT), measured in the horizontal plane, showed a non-significant increase, Lens Axial Height (LAH), measured in the vertical plane, significantly increased in both age groups. The correlation between reduced IOP and increased vertical lens dimensions (LAH) is consistent with previous reports. Reduced pressure within the anterior chamber and vitreous body has been associated with lens expansion and elongation [25,26]. Numerous studies in both human and veterinary medicine have documented IOP reductions following anaesthetic administration [25,27,28]. The decrease in IOP and increases in LT are thought to result from reduced aqueous and vitreous humour production or increased elimination of these fluids, thereby lowering intraocular volume. Such changes can lead to measurable structural alterations in corneal thickness, anterior chamber depth, and lens configuration [25,29]. Considering these findings, the fact that the sedation protocol used in our study caused changes in the anatomical structures of the eye is consistent with the data reported in the literature.

When sedated and non-sedated parameters were compared between juvenile and adult subgroups, no statistically significant differences were detected. However, when sedated and non-sedated values were compared within each age group, statistically significant differences emerged in several parameters. These within-group comparisons provided a more homogeneous age-based distribution, but studies with a larger sample size would more clearly reveal how the sedation protocol affects ocular parameters in Yellow-legged Gulls. Nonetheless, age-related physiological variability and inter-individual differences may have increased variance within the combined dataset, thereby influencing the statistical outcomes. In addition, minor variability associated with measurement techniques and individual stress responses may have contributed to the observed limitations. *Larus michahellis* is a sexually monomorphic species, and reliable morphological sexing is currently not possible. Molecular sexing methods were not applied in this study due to technical limitations and practical constraints. Some studies have reported morphometric measurements such as head, bill, wing, and tarsus lengths as potential indicators of sex [30], but geographic variation among populations and overlapping values between sexes make these predictions unreliable. To avoid inconsistent or speculative results, all data were analyzed without sex differentiation. This represents a limitation of the present study. Further studies with larger sample sizes and more homogeneous age groupings are warranted to clarify age-related effects and corroborate the present findings. The aim of this study was to evaluate the effects of intranasal butorphanol-diazepam sedation on tear production, intraocular pressure (IOP), and ocular biometry in Yellow-legged Gulls *(Larus michahellis)*. In our study, group classifications were performed to achieve as precise and homogeneous distributions as possible. We believe that each chemical agent may exert different effects in different species, and therefore, species-specific studies are one of the most important considerations. Consequently, it may provide preliminary data to support future reference studies with larger sample sizes, contributing to species-specific evaluation of ocular parameters.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (C.N.) on reasonable request.

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Research Article

The Effects of Urethane and Ketamine-Xylazine Anesthesia on Electromyographic Measurements in a Streptozotocin-Induced Diabetic Rat Model

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Abstract

Diabetes mellitus is a rapidly increasing global health concern, and neuropathy constitutes one of its significant complications. In animal models of diabetic neuropathy, invasive electromyography (EMG) is a widely applied approach. However, the choice of anesthetic agent represents a critical methodological factor, as it can directly modulate nerve conduction and muscle responses, thereby influencing the reliability of electrophysiological outcomes. This study investigated the comparative effects of ketamine-xylazine and urethane anesthesia on EMG parameters in streptozotocin-induced diabetic rats. Electrophysiological assessments of the gastrocnemius muscle demonstrated that urethane anesthesia produced markedly higher amplitudes and prolonged compound muscle action potential (CMAP) durations, potentially masking neuropathic deficits. In contrast, ketamine-xylazine anesthesia preserved the expected electrophysiological hallmarks of diabetic neuropathy, including reduced amplitudes and shortened CMAP durations. These findings indicate that urethane is not a pharmacologically inert anesthetic but one that may artificially alter neuromuscular transmission, leading to misleading interpretations in neuropathy models. Conversely, ketamine-xylazine provides more consistent results aligned with the established pathophysiology of diabetic neuropathy. In conclusion, the selection of anesthetic agent has profound implications for both the validity and translational relevance of electrophysiological research. Therefore, in preclinical neuropathy studies, ketamine-xylazine should be preferred over urethane as a more reliable and methodologically appropriate anesthetic protocol.

Keywords: Diabetes mellitus, Diabetic neuropathies, Streptozotocin, Wistar rat, Electromyography, Anesthesia, Ketamine, Xylazine, Urethane, Neuromuscular transmission

Introduction

Diabetic neuropathy is one of the most common and serious complications of diabetes mellitus, characterized by impaired nerve conduction, muscle weakness, and sensory deficits. To elucidate the underlying mechanisms, experimental animal models are employed, with the streptozotocin (STZ)-induced diabetic rat model being widely preferred due to its reproducibility and similarity to human pathophysiology. Electrophysiological methods, particularly electromyography (EMG) and compound muscle action potential (CMAP) recordings, are considered the gold standard for evaluating peripheral nerve conduction and neuromuscular function in these models.

In animal experiments, anesthesia is mandatory for performing invasive electrophysiological recordings. However, since the anesthetic agent used may directly or indirectly influence nerve conduction, synaptic transmission, and muscle responses, it is regarded as a methodologically critical variable [1]. The ketamine–xylazine combination is one of the most commonly used protocols in rodent studies, owing to its rapid onset of action and ability to provide adequate surgical depth. It has been reported that ketamine and xylazine affect glucose metabolism and cardiovascular stability [2-4] On the other hand, urethane is known for providing long-lasting and stable anesthesia, with relatively limited effects on cardiovascular and respiratory functions, and is therefore preferred in electrophysiological studies [5,6].

Nevertheless, urethane has been shown to exert direct effects on neurotransmitter systems, with evidence indicating its ability to modulate nicotinic acetylcholine receptor activity and acetylcholine release [7-9] This situation raises methodological concerns, particularly in

experimental designs where neuromuscular transmission is a primary parameter. Considering the increased sensitivity of diabetic animals to anesthetic agents, the selection of an appropriate anesthesia protocol is critically important not only for animal welfare but also for the reliability and reproducibility of the data obtained.

This study aims to provide a methodological contribution to the electrophysiological approaches used in diabetic neuropathy models by comparing the effects of ketamine-xylazine and urethane anesthesia on EMG recordings in STZ-induced diabetic rats.

Materials and Methods

Ethical Statement

This study was approved by the Manisa Celal Bayar University Local Ethics Committee for Animal Experiments (Approval date and number: 29/07/2025; 77.637.435/331).

Experimental Animals

A total of 18 male Wistar albino rats (22-24 weeks old, 350-400 g) were obtained from the institutional Experimental Animal Application and Research Center. The animals were housed under standard laboratory conditions (22±2°C, 50±10% humidity, 12/12 h light-dark cycle) with free access to water and standard pellet food. Rats were kept in polycarbonate cages containing corn-cob bedding, which was replaced once per week. Trained personnel monitored the animals daily for general health, activity, grooming, and hydration. All housing and husbandry procedures complied with institutional animal care guidelines and the ARRIVE recommendations.

Experimental Groups

The animals were randomly divided into four groups:

KetamineC (n=5): Control rats under ketamine-xylazine anesthesia

KetamineD (n=4): Diabetic rats under ketamine-xylazine anesthesia

UrethaneC (n=4): Control rats under urethane anesthesia

UrethaneD (n=5): Diabetic rats under urethane anesthesia

Diabetes Induction

In the KetamineD and UrethaneD groups, diabetes was induced after 6-8 h of fasting by a single intraperitoneal (i.p.) injection of streptozotocin (STZ; 60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) freshly prepared in 50 mM sodium citrate buffer (pH 4.5). To prevent acute hypoglycemia, the animals were provided with 10% sucrose solution for 48 h following the injection. Seventy-two h after the injection, blood glucose levels were measured from the tail vein (Accu-Chek Active, Roche, Germany), and animals with blood glucose levels > 300 mg/dL were considered diabetic [10]. Throughout the 4-week experimental period, the body weights and fasting glucose levels of the animals were recorded on a weekly basis.

Anesthesia Protocols

At the end of the fourth week, prior to electrophysiological recordings, the following anesthetic protocols were administered to the animals:

Ketamine groups (KetamineC, KetamineD): Ketamine 75 mg/kg + Xylazine 10 mg/kg, i.p.

Urethane groups (UrethaneC, UrethaneD): Urethane 1.5 g/kg, i.p.

The depth of anesthesia was assessed by pedal reflex, and rectal temperature was continuously monitored and maintained at approximately 36.5°C.

Electrophysiological Recordings

Under deep anesthesia, an incision of approximately 2.5 cm was made on the posterior surface of the right hind limb to expose the sciatic nerve. Bipolar hook electrodes (10 mm length, 0.35 mm diameter, 3 mm interelectrode distance) were placed on the nerve. The recording electrode (needle type) was inserted into the gastrocnemius muscle between the tendons, approximately 15 mm distal to the sciatic trifurcation. Electrical stimuli were delivered at supramaximal intensity with a duration of 0.2 msec, a frequency of 1 Hz, and an initial current of approximately 0.1 mA [11]. Electromyographic recordings were obtained using the LabChart 7 software (ADInstruments, Australia), and the following parameters were evaluated: latency (msec), amplitude (mV), and CMAP duration (msec).

At the end of the electrophysiological recordings, all animals anesthetized with urethane were humanely euthanized before regaining consciousness, in accordance with the institutional animal ethics approval and the AVMA Guidelines for the Euthanasia of Animals (2020). Urethane was used solely for acute terminal EMG procedures, and no survival or long-term follow-up was performed in urethane-treated rats.

Statistical Analysis

Data were presented as median (minimum-maximum). Normality was assessed using the Shapiro-Wilk test. The Kruskal-Wallis test was employed for comparisons among groups, and the Mann-Whitney U test was used for pairwise comparisons. Values of $P<0.05$ were considered statistically significant. Statistical analyses were performed using SPSS version 30.0 software (IBM Corp., Armonk, NY, USA). A post-hoc power analysis was performed in G*Power 3.1 using the CMAP duration data from diabetic

rats anesthetized with Ketamine (n=4) and urethane (n=5). The standardized effect size for the difference between these groups was calculated as Cohen's d=2.5567. Using a two-tailed α=0.05, the achieved power of the study was 0.90.

Results

Demographic Characteristics of the Experimental Diabetic Groups

The demographic evaluation of the experimental diabetic groups revealed that both KetamineD and UrethaneD animals exhibited persistent hyperglycemia throughout the study period. On day 3 following STZ induction, mean blood glucose levels were markedly elevated in both groups (KetamineD: 600.00±0.00 mg/dL; UrethaneD: 484.20±76.06 mg/dL). During the first week, glucose values remained high, with the UrethaneD group showing slightly higher mean levels (558.00±93.91 mg/dL) compared to the KetamineD group (449.50±42.68 mg/dL). By weeks 2–4, both groups sustained glucose concentrations above the diabetic threshold, with mean values exceeding 500 mg/dL across all time points (Week 2: 582.00±20.78 vs. 575.20±55.45 mg/dL; Week 3: 514.00±53.63 vs. 550.20±45.73 mg/dL; Week 4: 523.25±91.84 vs. 591.40±19.23 mg/dL, for KetamineD and UrethaneD respectively).

In terms of body weight, both groups demonstrated a gradual reduction following STZ administration, consistent with the diabetic phenotype. The mean body weight of KetamineD rats decreased from approximately 370 g at baseline to ~314 g at week 4, whereas UrethaneD rats declined from ~345 g to ~324 g in the same period. These findings confirm the successful establishment of experimental diabetes in both groups and indicate comparable metabolic alterations across anesthetic regimens.

As shown in *Table 1*, significant overall differences were observed among the groups in terms of latency, amplitude, and CMAP duration. The urethane-anesthetized groups exhibited shorter latencies and higher amplitude values compared with the ketamine groups. In addition, CMAP durations tended to be longer in the urethane groups, particularly in diabetic animals, whereas the shortest durations were observed in the ketamine diabetic group.

As shown in *Table 2*, the KetamineC group exhibited a significantly longer CMAP duration compared with the KetamineD group, accompanied by an extremely large effect size. In addition, latency values in the UrethaneD group were significantly higher than those in both the KetamineC and UrethaneC groups. A significant difference was also observed between UrethaneD and UrethaneC, where UrethaneD demonstrated markedly higher latency with a very large effect size. Furthermore, the latency comparison between KetamineD and UrethaneC was significant and associated with a very large effect size. Although the remaining comparisons were not statistically significant, several displayed large effect sizes, suggesting substantial anesthetic-related differences.

As shown in *Fig. 1*, individual CMAP latency values demonstrated a clear separation between groups. Ketamine-treated control rats (KetamineC) exhibited

Table 1. *Electrophysiological parameters of experimental groups*

EMG Parameter	KetamineC Median (Min-Max)	KetamineD Median (Min-Max)	UrethaneD Median (Min-Max)	UrethaneC Median (Min-Max)	P Value
Latency (msec.)	1.25 (1.25-1.75)	2.00 (1.00-2.25)	1.75 (1.50-2.25)	0.88 (0.75-1.00)	0.015
Amplitude (mV)	14.05 (11.09-21.09)	15.25 (9.60-19.62)	22.46 (18.39-41.22)	24.44 (19.02-33.53)	0.016
CMAP duration (msec.)	4.50 (4.00-4.75)	2.13 (1.75-2.50)	3.00 (2.75-4.00)	3.13 (2.75-4.00)	0.003

Values are presented as median (minimum-maximum). KetamineC: Ketamine control (n=5), KetamineD: Ketamine diabetic (n=4), UrethaneC: Urethane control (n=4), UrethaneD: Urethane diabetic (n=5). Statistical analysis was performed using the Kruskal–Wallis test. P<0.05 was considered statistically significant.

Table 2. *Bonferroni-adjusted pairwise comparisons and effect sizes (Cohen's d) for electrophysiological parameters among experimental groups*

Comparison	Parameter	Adj p	Cohen's d
KetamineC vs KetamineD	Latency	1.000	−1.03
	Amplitude	1.000	0.07
	CMAP duration	0.001	7.86
KetamineC vs UrethaneD	Latency	0.023	−1.43
	Amplitude	0.091	−1.40
	CMAP duration	0.255	3.19
KetamineC vs UrethaneC	Latency	0.560	2.71
	Amplitude	0.142	−1.89
	CMAP duration	0.393	2.93
KetamineD vs UrethaneD	Latency	1.000	0.03
	Amplitude	0.204	−1.36
	CMAP duration	0.405	−2.56
KetamineD vs UrethaneC	Latency	0.041	2.32
	Amplitude	0.204	−1.83
	CMAP duration	0.255	−2.53
UrethaneD vs UrethaneC	Latency	0.023	3.51
	Amplitude	1.000	−0.02
	CMAP duration	1.000	−0.10

Median (min-max) values are reported for each pairwise group comparison. Adjusted P-values represent Bonferroni-corrected Mann-Whitney U post-hoc tests, and values of P<0.05 were considered statistically significant. Effect sizes (Cohen's d) were calculated using the SPSS "Independent Samples Effect Sizes" module based on pooled standard deviations. Positive d values indicate higher parameter values in the first group of each comparison, whereas negative d values indicate higher values in the second group

relatively lower and more clustered latency values, whereas diabetic rats under ketamine anesthesia (KetamineD) showed a wider distribution with generally higher latencies. In the urethane-anesthetized diabetic group (UrethaneD), latency values remained elevated and displayed greater variability compared with controls. In contrast, urethane-treated control rats (UrethaneC) presented the lowest and most narrowly distributed latencies among all groups.

As shown in *Fig. 2*, individual CMAP amplitude values exhibited a distinctly different distribution across the groups. The ketamine-treated control group (KetamineC) displayed lower amplitudes with a narrow distribution range. The diabetic ketamine group (KetamineD) showed similarly low amplitudes, although the variability was slightly greater. Diabetic rats evaluated under urethane anesthesia (UrethaneD) demonstrated higher and more widely distributed amplitude values, clearly separating them from the ketamine groups. The highest amplitude values were observed in the urethane-treated control group (UrethaneC), which appeared markedly different from all other groups in terms of both amplitude magnitude and distribution range.

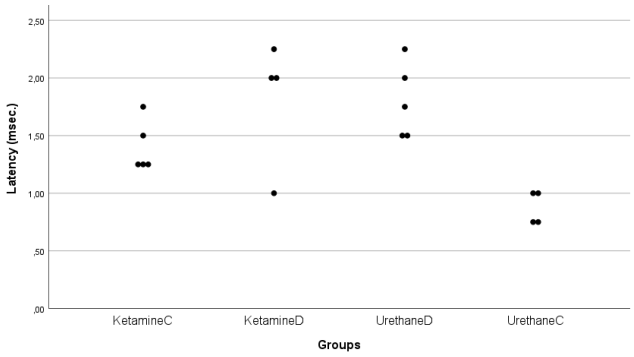

Fig 1. Individual CMAP latency values across experimental groups. KetamineC: Ketamine-Control; KetamineD: Ketamine-Diabetic; UrethaneD: Urethane-Diabetic; UrethaneC: Urethane-Control

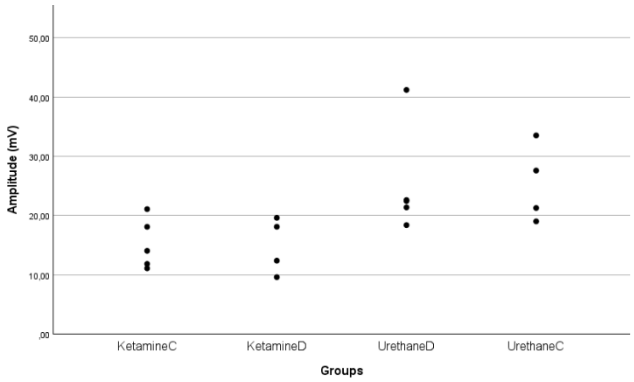

Fig 2. Individual CMAP amplitude values across experimental groups. KetamineC: Ketamine-Control; KetamineD: Ketamine-Diabetic; UrethaneD: Urethane-Diabetic; UrethaneC: Urethane-Control

As shown in *Fig. 3*, individual CMAP duration values exhibited a distinct distribution pattern across the groups. The ketamine-treated control group (KetamineC) displayed the highest CMAP durations, with values tightly clustered within a narrow range. In contrast, the diabetic ketamine group (KetamineD) showed markedly lower CMAP durations, with values clustered at the lower end of the distribution. Diabetic rats evaluated under urethane anesthesia (UrethaneD) presented moderate CMAP durations, with a broader distribution compared to the ketamine groups. The urethane-treated control group (UrethaneC) demonstrated a distribution pattern similar to that of the UrethaneD group, with CMAP durations remaining within a moderate range.

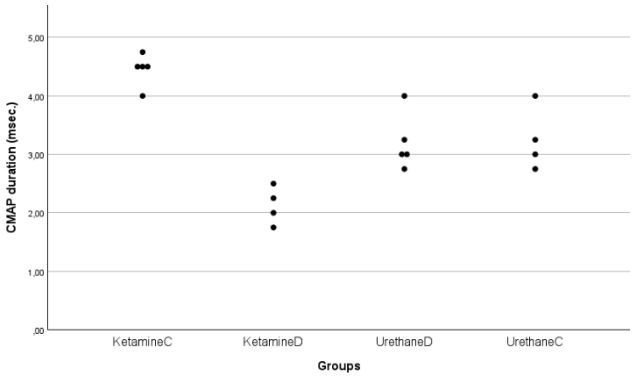

Fig 3. Individual CMAP duration values across experimental groups. KetamineC: Ketamine-Control; KetamineD: Ketamine-Diabetic; UrethaneD: Urethane-Diabetic; UrethaneC: Urethane-Control

Discussion

In this study, the effects of different anesthesia protocols on electrophysiological measurements were investigated in STZ-induced diabetic rats. Our findings demonstrate that ketamine-xylazine anesthesia revealed the expected neuropathic patterns, whereas urethane anesthesia significantly altered the compound muscle action potential (CMAP) parameters. In particular, the marked increase in amplitude and the prolongation of CMAP duration were found to have the potential to mask neuropathic impairments. This suggests that urethane may lead to misleading electrophysiological outcomes in disease models.

Urethane is frequently preferred in electrophysiological studies due to its ability to provide long-lasting and stable anesthesia, along with its relatively limited effects on cardiorespiratory functions [7,12]. However, it is not a pharmacologically inert agent. Studies in the literature have demonstrated that urethane enhances GABAergic transmission, suppresses glutamatergic signaling, and may increase nicotinic receptor activity within the cholinergic system through acetylcholinesterase inhibition [7-9,13-15]. These pharmacological properties may account for

the increase in CMAP amplitude and the prolongation of duration observed in our study. Furthermore, a recent investigation demonstrated that urethane and alternative agents produced distinct transmission profiles in visual system electrophysiology, showing that the choice of anesthetic exerts a significant impact on signal amplitude and temporal dynamics [16,17]. This finding supports the notion that urethane may artificially alter electrophysiological outcomes from a methodological perspective.

Urethane has also been reported to modify endocrine function, including an increase in plasma insulin levels that become evident approximately 20 min after administration in rats [18,19]. In the present study, however, urethane was administered as a single terminal bolus, and EMG recordings were completed within approximately 5-6 min of injection, with each individual recording lasting about 1 min. Thus, the time window during which electrophysiological data were acquired is likely to precede or only minimally overlap with the delayed endocrine effects described in that study. Moreover, diabetic neuropathy is a chronic complication, and it remains uncertain to what extent a short-lived, acute change in circulating insulin could rapidly modify nerve or muscle electrophysiology within a few minutes. Taken together, these considerations suggest that, while subtle metabolic influences cannot be fully excluded, they are unlikely to be the primary driver of the differences observed between anesthetic protocols. Instead, our findings are more consistent with the direct neurophysiological actions of urethane on synaptic transmission and membrane excitability.

The NMDA receptor antagonism of ketamine and the sedative and analgesic effects of xylazine through α2-adrenergic agonism have made this combination one of the most widely used protocols in experimental neurophysiology [20]. Several studies have reported that ketamine-xylazine anesthesia has minimal effects on peripheral nerve conduction and CMAP recordings [2,3,5]. In our study, the low amplitudes and short CMAP durations observed in the ketamine groups were found to be consistent with the expected electrophysiological pattern of diabetic neuropathy [21]. In addition, the literature has reported that in STZ-diabetic rats, sufficient anesthetic depth could not be achieved with ketamine + xylazine or medetomidine + ketamine, and that diabetes increases anesthetic sensitivity [22]. In one study, it was shown that the induction time with ketamine-xylazine was shortened in diabetic rats; however, this difference was not associated with glucose level or body weight [23]. These data suggest that ketamine-xylazine can preserve electrophysiological reliability even in diabetic models.

Diabetic neuropathy is characterized by reduced conduction velocity, prolonged latency, and decreased amplitude [24]. While these alterations were observed in the ketamine group in accordance with the literature, the increase in amplitude and prolongation of duration seen in the urethane group may present neuropathic impairment as less severe than it actually is. This methodological issue complicates the interpretation of findings in diabetic neuropathy studies and may lead to conflicting results across laboratories.

CMAP measurements are widely used objective parameters not only in experimental neuropathy models but also in clinical studies [25]. Therefore, the significant alteration of electrophysiological outcomes by urethane represents a methodological risk in the translation of preclinical data to human disease. Our findings indicate that the ketamine–xylazine protocol is more appropriate in terms of translational reliability.

In conclusion, this study demonstrates that urethane anesthesia can influence electrophysiological outcomes in diabetic rats and may lead to changes in CMAP parameters that partially obscure neuropathic alterations. Our findings indicate that, particularly in disease models where subtle neuropathic changes must be assessed with precision, electrophysiological data obtained under urethane anesthesia should be interpreted with caution. In contrast, the ketamine–xylazine combination provides more consistent and interpretable CMAP results in this experimental model. Nevertheless, further studies with larger sample sizes and additional physiological parameters are needed to more clearly determine the comparative suitability of these anesthetic agents in diabetic neuropathy research.

This study has several limitations. First, only a diabetic neuropathy model was used, and it remains unknown whether urethane exerts similar electrophysiological effects in neuropathies induced by chemotherapy, trauma, or other systemic diseases. Second, receptor-level or molecular analyses that could directly clarify the pharmacological actions of urethane were not performed. Third, while electrophysiological alterations in CMAP parameters support the presence of neuropathic involvement, EMG findings alone cannot definitively confirm diabetic neuropathy. Comprehensive confirmation would require additional assessments such as sensory nerve conduction studies, motor unit number estimation (MUNE), intraepidermal nerve fiber density measurements, or behavioral pain evaluations. Fourth, the total number of animals used in this study was relatively small, and the group sizes were slightly unbalanced, which may limit the generalizability of the findings. Although a post-hoc power analysis (Cohen's $d=2.5567$; $1-\beta=0.90$) demonstrated adequate statistical strength for the primary electrophysiological comparison, the exploratory pilot nature of the study requires cautious

interpretation. Larger future studies with prospectively calculated sample sizes are necessary to validate and expand these observations. Fifth, advanced physiological monitoring (e.g., HR, MAP, SpO_2, $EtCO_2$, EEG/BIS) was not performed. Reflex-based assessments were used, but the lack of continuous cardiorespiratory data limits the precision with which anesthetic depth can be interpreted. Future studies incorporating invasive or non-invasive physiological monitoring systems may better characterize the relationship between anesthetic depth and EMG outcomes.

In future research, combining pharmacological antagonists with neurotransmitter analyses may provide deeper insight into the neural mechanisms underlying urethane's electrophysiological effects. Furthermore, the inclusion of additional electrophysiological assessments such as sensory nerve potentials or MUNE could strengthen methodological robustness. Considering the markedly elevated amplitude values observed in the urethane group, future studies may also explore whether urethane has potential applications in investigating -or possibly mitigating-muscle damage associated with neuropathy.

Declarations

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

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Declaration of Generative Artificial Intelligence (AI): No generative AI tools were used to create or write the scientific content, tables, figures, or analyses presented in this manuscript. AI-based tools (OpenAI) were used solely to improve the clarity and readability of the language. Reference organization was performed using Mendeley software.

Author Contributions: B.M. contributed to the study conception and design, development of the experimental protocol, execution of EMG recordings, data analysis, and drafting of the manuscript. E.K. contributed to animal care and daily monitoring, diabetes induction, administration of anesthesia, and technical assistance during electrophysiological procedures, as well as the critical revision of the manuscript for important intellectual content. Both authors contributed to the interpretation of the results, reviewed the manuscript critically, and approved the final version of the article. The authors affirm that all listed contributors meet the journal's Authorship Rights and Ethical Principles, and no individuals other than the authors contributed to the scientific content of this study.

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Research Article

Outcome of Gap Arthroplasty in Cats with Temporomandibular Joint Ankylosis: A Retrospective Study

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Abstract

This retrospective study describes the clinical features, imaging results, surgical management and long-term outcomes of gap arthroplasty (GA) in cats diagnosed with temporomandibular joint (TMJ) ankylosis. The medical data including signalment, clinical presentation, CT findings, surgical management, and follow-up results was obtained from previous patient records between 2020 to 2024. The mean age of the cats was 23.3 months. Of the 10 cats included, six were male and four were female. The study population consisted of four Domestic Shorthair cats, four Scottish Folds, and one cat each of the British Shorthair and British Longhair breeds. Trauma was the primary cause of ankylosis. Unilateral involvement was more common than bilateral, predominantly affecting the left joint. GA surgery was achieved using conventional surgical instruments rather than piezoelectric surgical devices. Bilateral GA was performed in two cats with bilateral ankylosis, while two other cats required contralateral surgery after recurrence. Immediate postoperative improvement in mandibular motion was achieved in all cases. Outcomes were classified as excellent in 3 cats, good in 4 cats, fair in 1 cat, and excellent after a second procedure in 2 cats that initially had poor outcomes. Owner-reported quality of life markedly improved at a mean follow-up of 16.8 months. Despite the lack of postoperative imaging, clinical outcomes were consistently favorable. According to results of this study, GA remains a reliable, accessible, and cost-effective surgical option, particularly in cats with advanced ankylosis or poor body condition.

Keywords: Arthroplasty, Cat, Computed tomography, Malocclusion, Temporomandibular ankylosis

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Introduction

The temporomandibular joint (TMJ) is a synovial condylar articulation between the condyloid process of the mandible and the mandibular fossa of the temporal bone and provides the hinge-like motion required for mouth opening and closing. In dogs, slight laterotrusion is possible, whereas in cats, lateral mandibular movement is minimal because of the restrictive morphology of the joint [1-3].

Fractures, tearing, contracture, fibrosis of periarticular soft tissues, luxation, congenital malformations, infections and neoplasia may result with dysfunction of the TMJ and further with intra-articular (true ankylosis) and/or extra-articular ankylosis (false-pseudoankylosis) [4-7]. Traumatic lesions that may lead to ankylosis include luxation of the condylar process with or without fractures of the condylar process, mandibular fossa, retroarticular process, coronoid process, or zygomatic arch [8,9]. Additionally, it has been noted that TMJ ankylosis in cats, both intra-articular and extra-articular components' osseous bridging and degenerative joint changes as well as complex fractures involving the mandibular fossa, is usually results in permanent ankylotic fusion [10].

TMJ ankylosis has severe clinical consequences, impairing feeding, grooming, thermoregulation, and drinking, and may cause malnutrition, malocclusion, periodontal disease, and mucosal ulceration [2,5,11,12]. In cats, tongue entrapment and life-threatening airway obstruction may occur due to severe ankylosis. Diagnosis relies on clinical signs, such as restricted mandibular motion, malocclusion and facial asymmetry, supported by advanced imaging. Computed tomography (CT) and cone-beam CT (CBCT) are considered the gold standards, with multiplanar and 3D

reconstructions providing detailed lesion characterization and guiding the surgical planning [13-17].

Surgical intervention is the treatment of choice in most of the cases with TMJ ankylosis. Although conservative management consists of stretching maneuvers combined with periarticular corticosteroid administration have been described as a means of improving mandibular range of motion and relieving discomfort, this merely postpones the need for surgery. Nonetheless, most cases require surgical intervention, even if they initially respond to conservative therapy [12,18].

Surgical treatment is considered the preferred method in cases where the clinical course is severe, or conservative treatment does not provide sufficient functional improvement. Reported techniques include gap arthroplasty (GA), interpositional arthroplasty (IA), excisional ostectomy, and segmental mandibulectomy (SM) [5,18-21]. IA techniques have employed autogenous tissues such as temporal muscle, fascia, or fat, or synthetic materials such as silicone and PTFE [22,23]. Among these, gap arthroplasty remains the most widely described and continues to be the preferred technique in severe cases, as it restores mandibular motion by creating a functional gap between the condylar process and the temporal bone [11]. Despite being invasive, GA has been associated with favorable functional outcomes when combined with appropriate postoperative care and physiotherapy [11,18,21]. In humans, gap arthroplasty also has been demonstrated to provide satisfactory long-term functional results for temporomandibular joint (TMJ) ankylosis [24].

This study reports the clinical and CT features, surgical technique, and outcomes of gap arthroplasty in cats with extensive TMJ ankylosis, contributing to species-specific outcome data to guide surgical decision-making.

Material and Methods

Ethical Statement

The study was approved by Animal Experiments Local Ethics Committee of Ankara University on 23/07/2025 under decision number 2025-14-156. An "Informed Consent Form" was obtained from the animal owners before examination of animals.

Case Inclusion

The patient recording system of the Ankara University Faculty of Veterinary Medicine Animal Hospital was retrospectively reviewed, the cats with a diagnosis of TMJ ankylosis treated by GAP arthroplasty between January 2020 and December 2024 were included in the study. The cats which have missing information of medical records including anamnesis, clinical findings, radiographic examination and minimum follow up time of 6 months were excluded from the study.

Medical Records

Information was obtained from the medical records including signalment, anamnesis, physical examination, laboratory analyses, diagnostic imaging results, surgical treatment, post-operative care and outcome. Follow-up information on the cases were obtained from medical records and telephone interviews with owners.

Computed Tomography

Spiral computed tomography (CT) examinations were performed using a 32-slice CT scanner (Supria model, Fujifilm Healthcare Corporation, Japan) available at the Ankara University Faculty of Veterinary Medicine, Department of Radiology. All cats were positioned in sternal recumbency (head-first) and scanned under general anesthesia. Acquisition parameters included 120 kV, 300 mAs, and a slice thickness of 1.25 mm. For image evaluation, a window level of 750 HU and a window width of 3500 HU were applied. Anesthesia induction was achieved with intravenous (IV) administration of butorphanol (0.1 mg/kg), followed by diazepam (0.3 mg/kg) and propofol (4 mg/kg). Due to restricted mouth opening and short scanning duration, none of the cats were intubated during CT imaging. Three-dimensional reconstructions of the images used for publication were created using OsiriX Lite DICOM software.

Surgical Technique

Anesthesia was maintained as described in the CT protocol. To facilitate intubation, gentle manual traction was applied to the mandibular and maxillary premolar teeth using gauze bands until sufficient oral opening allowed placement of the laryngoscope. Tracheostomy was required in only one case. Anaesthesia was maintained with isoflurane in oxygen, and cefazolin sodium (25 mg/kg IV) was administered perioperatively. Continuous infusion of butorphanol-ketamine in 0.9% NaCl (3-5 mL/kg/hour) provided intraoperative analgesia. Cats were placed in lateral recumbency with their heads slightly elevated. As previously described [11], a full-thickness incision was made along the ventral zygomatic arch using a lateral approach, and the periosteum was elevated to expose the zygomatic arch, coronoid and condylar processes, and mandibular fossa. The tissues were retracted with a Hohmann retractor or a periostal elavator, later zygomectomy, coronoidectomy and condylectomy were performed using an osteotome and mallet *(Fig. 1)*. Recontouring of the mandibular fossa (fossectomy) was accomplished using a rongeur, and all remaining surface irregularities were meticulously smoothed with a bone file to obtain a uniform and anatomically congruent articular gap. The area was irrigated with saline and closed in a routine manner. In cases when CT imaging revealed bilateral ankylosis, bilateral gap arthroplasty was performed

during the same operative session. Contralateral GA was performed, if postoperative re-ankylosis occurred.

Post-Operative Care and Follow up

Postoperatively, amoxicillin-clavulanate (Klamoks® BID 200 mg-28 mg/5mL, Bilim) (22.5 mg/kg PO, q12h) was administered for 7 days. For analgesia, meloxicam (Metacam®, 0,5 mg/mL, Boehringer Ingelheim) (0.05 mg/kg PO, q24h) and tramadol (Contramal®, 100 mg/mL, Abdi İbrahim) (2 mg/kg PO, q12h) were prescribed for 5 days. In addition, a physiotherapy regimen consisting of gentle stretching exercises using a toy or chewing treat, together with massage of the masticatory muscles, was recommended to the owners to facilitate recovery and improve mandibular mobility.

Postoperative follow-up was performed by routine clinical examinations on days 5 and 10 after surgery. Quantitative measurement of maximum mouth opening could not be performed due to the retrospective nature of the study and because standardized measurements were not routinely recorded in the clinical setting. This is acknowledged as a limitation of the study. Instead of the quantitative measurements, postoperative recovery was primarily assessed clinically and through owner observations of feeding behavior, chewing ability, and overall comfort.

Postoperative CT or radiographic imaging was not performed because it required general anesthesia, involved additional financial cost, and most owners declined further imaging once satisfactory clinical improvement was achieved. Therefore, follow-up assessment relied primarily on clinical examination and owner observations.

Follow-up telephone interviews were conducted with the owners 6 months postoperatively and again at the time of manuscript preparation to assess long-term outcomes, including appetite, food intake, and recurrence of clinical signs.

Assessment of the Outcome

Outcome categories (excellent, good, fair, poor) were assigned based on postoperative mandibular mobility, feeding ability, and recurrence, which is in accordance with previously described evaluation systems [5,11,18] in feline temporomandibular joint ankylosis *(Table 1)*. Owner-reported quality of life was also included in the assessment.

Results

During the defined study period, examination of medical records revealed that 13 cats underwent gap arthroplasty for the treatment of TMJ ankylosis. Three of these cats were excluded from the study due to missing medical records, leaving a total of 10 cases that met the inclusion criteria and were evaluated in this study.

The mean age of the cats was 23.3 months (range, 4-72 months). Of the 10 cats included, six were male and four were female. Among the males, four were neutered and two were intact, while among the females, two were spayed and

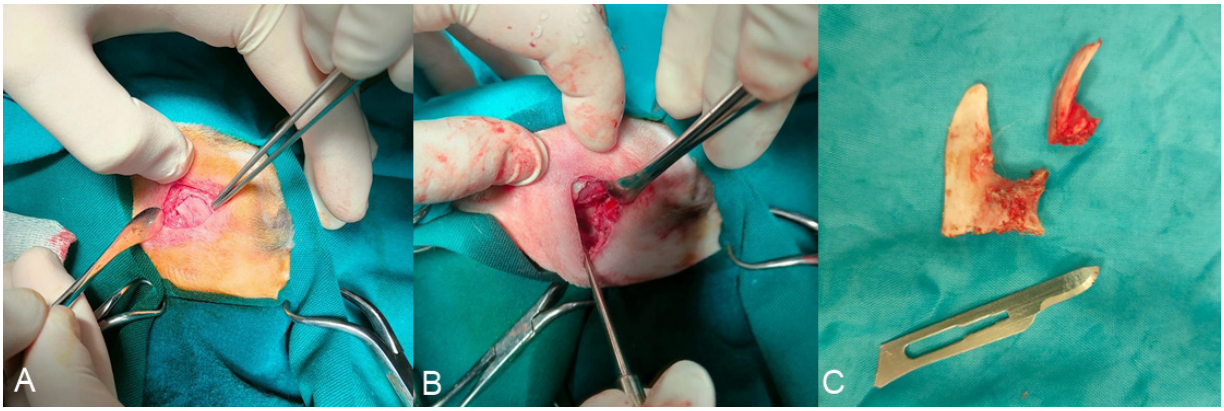

Fig 1. Intraoperative images illustrating the surgical procedure in Case 7. A: Appearance of the zygomatic arch following soft tissue retraction, B: View of the underlying soft tissues after zygomectomy, C: Resected condylar and coronoid processes removed en bloc with the zygomatic arch

Table 1. *Grading of the outcome of the GAP arthroplasty*

Outcome Grade	**Criteria**
Excellent	Normal feeding and grooming behaviors, acceptable mouth opening, and no recurrence or complications
Good	Near-normal function with only minor difficulties, absence of clinically relevant recurrence, and complications manageable with medical treatment
Fair	Mild-to-moderate dysfunction, such as partial dependence on soft diets or intermittent signs suggestive of recurrence
Poor	No significant improvement, or when recurrence or major complications required additional surgical intervention

two remained intact. With respect to breed distribution, the study population consisted of four Domestic Shorthair cats, four Scottish Folds, and one cat each of the British Shorthair and British Longhair breeds. The mean body weight of the patients was 2.07 kg (median, 1.8 kg; range, 1.0-3.2 kg).

Of the 10 cats included, nine had a documented history of trauma, whereas in one cat temporomandibular joint ankylosis developed in the absence of any known traumatic event. The interval between trauma and admission to clinic ranged from 15 days to 12 months, with a median of 2.25 months (mean, 3.05 months). Although the exact onset of clinical signs could not be determined in most cases, owners generally reported seeking veterinary care once complete loss of jaw mobility was observed. According to the available information, the interval between the perceived onset of complete loss of the TMJ function and presentation to the hospital varied from 3 days to 1 month. According to the medical records, in six cats at least one previous attempt to relieve the ankylosis had been performed at another clinic prior to referral. These procedures consisted of forced mouth opening under general anesthesia, sometimes combined with intra-articular injection of corticosteroids and local anesthetic agents. However, only transient improvement was achieved, and all cats subsequently developed recurrence of jaw immobility.

On clinical examination, all cats presented with severe restriction of mandibular mobility. Preoperatively, the interincisal distance was minimal, permitting only limited extension of the tongue for water or soft food intake, while normal prehension and mastication were impossible. Additional common clinical findings included hypersalivation, low body condition associated with impaired food intake, and poor grooming, particularly around the head and neck region. Furthermore, mandibular brachygnathia was noted in one cat (Case 9), and in another three cats (Case 1, 4 and 7) asymmetric malocclusion (wry bite) was observed *(Fig. 2, Fig. 3)*. Unilateral facial paralysis was also noted in one cat (Case 8).

Hematology and serum biochemistry results were heterogeneous and did not reveal a consistent pattern. Mild leukopenia and mild hypoalbuminemia were observed in five cats, while the remaining values were within reference limits.

In four cats, unilateral intra-articular ankylosis was identified, whereas in another four cats unilateral ankylosis involved both intra- and extra-articular structures *(Fig. 4)*. In two cats (Case 2 and 4), bilateral ankylosis with combined intra- and extra-articular involvement was present *(Fig. 5)*. All but one (Case 8) unilateral cases affected the left temporomandibular joint.

Among eight cats diagnosed with unilateral TMJ ankylosis, unilateral gap arthroplasty was performed in six cases. In the remaining two (Case 7 and 9), surgery was subsequently indicated for the contralateral joint because of the recurrence of the ankylosis after the initial procedure. Although no postoperative CT was available in these cases, clinical examination and deep palpation of the initial surgical site showed an enough GAP between the ostectomy lines. Depending on these signs, contralateral

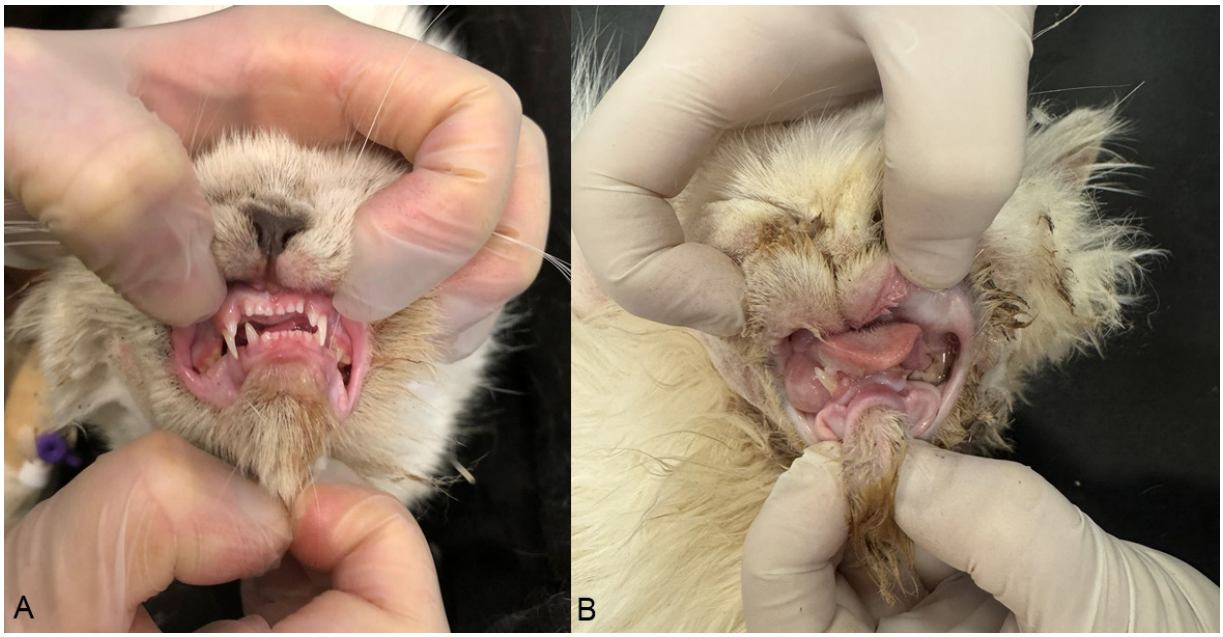

Fig 2. Clinical appearance of the assymetric malocclusion. A: Assymetric malocclusion in a Scottish Fold cat with a left shift of the mandible (Case 7), B: Assymetric malocclusion in a British Short Hair cat with a right shift of the mandible, and poor grooming is obvious around mouth and neck region (Case 4)

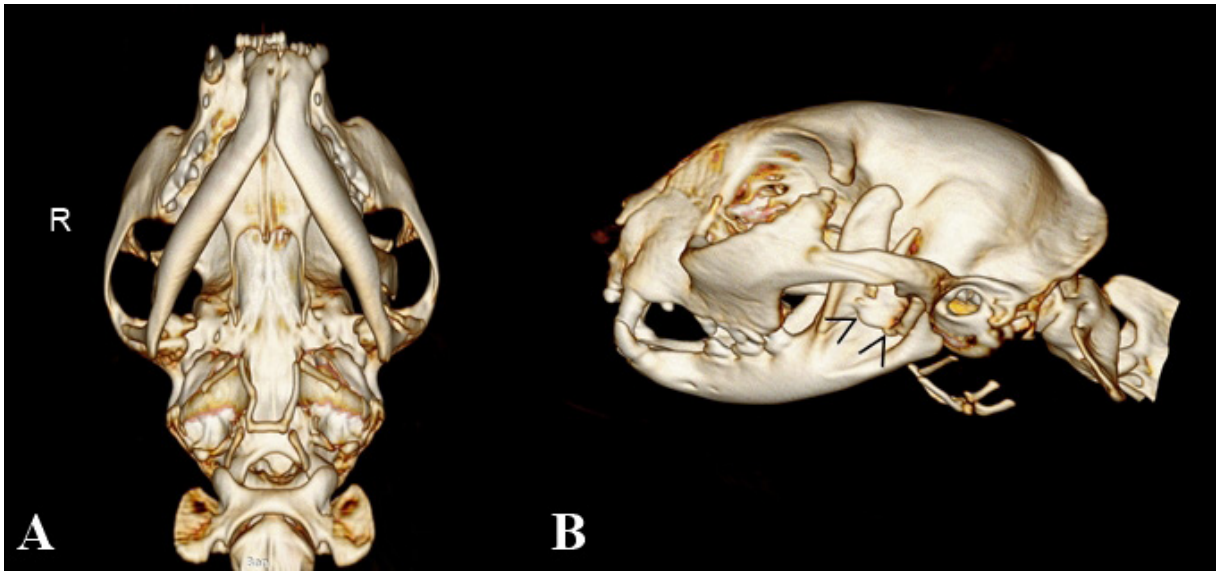

Fig 3. Three dimentional volume rendering CT images of the left side TMJ ankylosis (Case 1). A: Assymetric malloclusion (wry bite) with a left shift of mandible, B: Osseos proliferation and bridging (*arrowheads*)

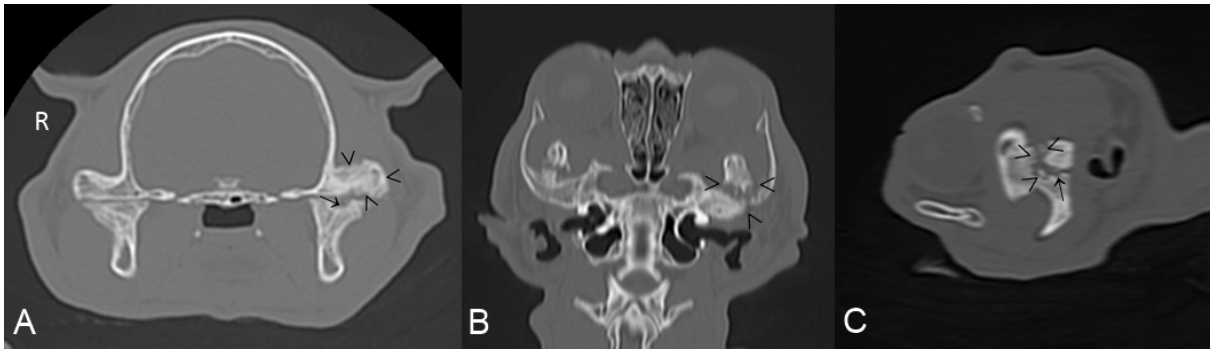

Fig 4. Transverse (A), dorsal (B) and left sagittal (C) CT images of the TMJ showing marked thickening and irregular osseous proliferation of the condylar process and mandibular fossa (*arrows and arrowheads*) (Case 3)

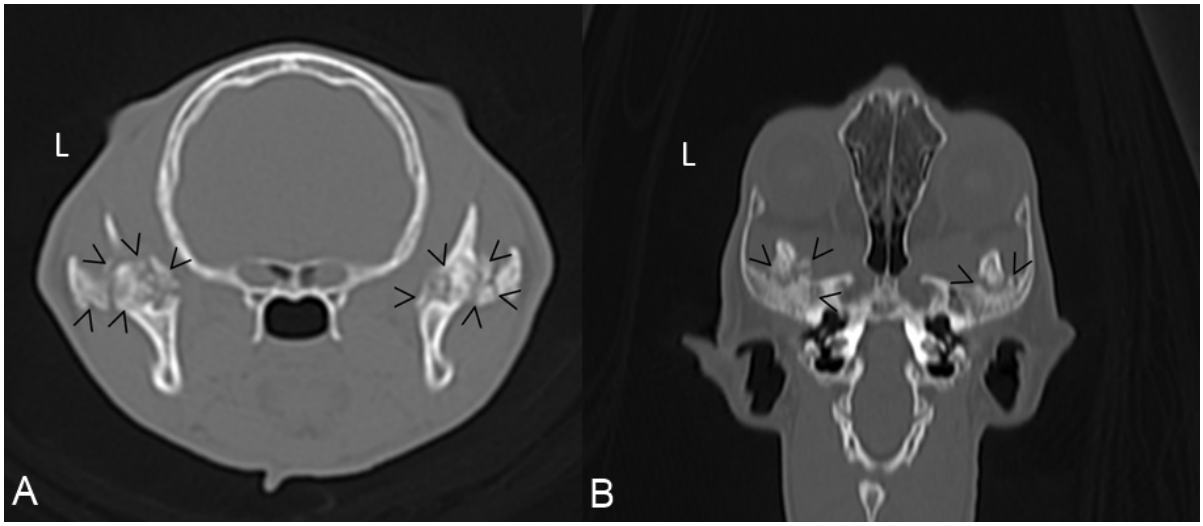

Fig 5. Transverse (A) and dorsal (B) CT images showing bilateral TMJ ankylosis (Case 4). Extensive osseous proliferation and bridging are present between the mandibular condyles and the temporal bones (*arrowheads*), resulting in loss of normal joint space and marked thickening of periarticular bone

surgery was decided. Bilateral GA in the same session was performed in 2 cats with bilateral TMJ ankylosis confirmed by CT *(Fig. 6)*.

Satisfactory range of motion at the level of TMJ was achieved in all cases immediately after GA. Suture removal was uneventful in all cats except one (Case 10), in which mild dermatitis developed due to early removal of the Elizabethan collar; the lesion resolved after two sessions of therapeutic laser treatment.

The outcome was classified as excellent in three cases (Cases 1, 2, and 4), good in four cases (Cases 3, 5, 8, and 10), and fair in one case (Case 6). In the remaining two cases (Cases 7 and 9), the initial outcome was poor following the first GA but became excellent after the contralateral procedure *(Fig. 7)*. One cat died seven months postoperatively due to panleukopenia, while the remaining nine were still alive at the time of manuscript preparation. The mean follow-up duration was 16.8 months (range, 7-33 months; median, 15 months). Detailed data of the cases is given in *Table 2*.

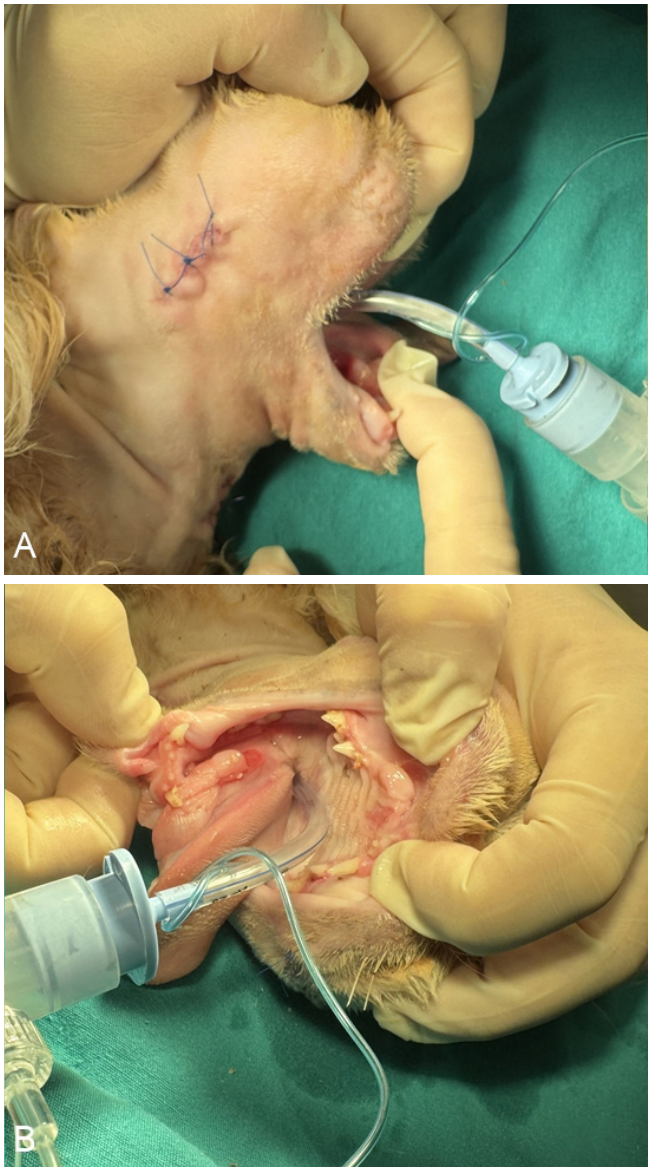

Fig 6. Immediate postoperative clinical appearance of Case 4, where bilateral gap arthroplasty was performed

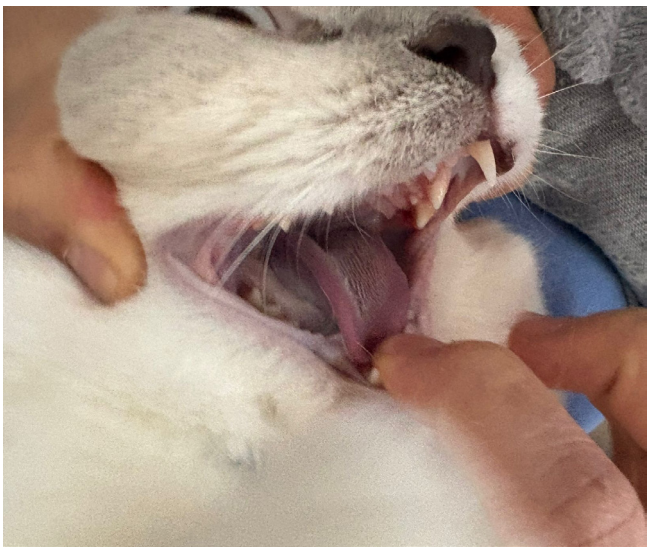

Fig 7. This image, taken by the owner six months after the second gap arthroplasty, demonstrates excellent mouth opening without any functional restriction in Case 7

The quality of life (QoL) reported by the owners was assessed through follow-up telephone interviews. According to the owners, all cats showed a marked improvement in terms of living comfort. The owner of the cat with a moderate outcome (Case 6) reported only a moderate improvement in quality of life; it was learned that the cat continued to eat soft food but that its condition was stable. The owner declined further reassessment or surgical intervention. Other owners reported their cats› post-operative recovery and quality of life as good to very satisfactory.

Discussion

This retrospective study evaluated the clinical features, imaging findings, surgical management, and long-term outcomes of GA in ten cats diagnosed with TMJ ankylosis. The results demonstrated that GA effectively restored mandibular mobility and improved quality of life in most cases. These findings reaffirm that GA remains a reliable and accessible treatment for feline TMJ ankylosis, particularly in cases where functional impairment is severe and conservative management fails.

Trauma was identified as the primary cause of TMJ ankylosis in most cats, consistent with previous reports emphasizing post-traumatic etiology [8,11]. Most of the affected cats were young adults, a finding also reported by Aghashani et al. [11], likely reflecting the higher risk of accidental trauma in active cats. Also, as it is stated in the previous studies, this may be related with the higher growth rate of the young individuals which make

Table 2. *Detailed data of the cases with temporomandibular joint (TMJ) ankylosis*

No.	Signalment	History	Clinical Findings	CT Imaging Findings	Bilateral/ Unilateral	Outcome/Follow up
1	DSH, neutred male, 4 y 1.5 m, 2.8 kg	Trauma; fall from a height	TMJ ankylosis, mandibular deviation	Left extra-intra ankylosis, asymmetric malocclusion	Unilateral	Excellent/11 m
2	SF, intact male, 1 y, 1.8 kg	Trauma; fall from a height	TMJ ankylosis, gingivitis	Bilateral extra-intra ankylosis	Bilateral	Excellent/21 m
3	BLH, intact male, 1 y 10 m, 3.1 kg	Trauma; fall from a height	TMJ ankylosis, dehydration, gingivitis	Left extra-intra ankylosis	Unilateral	Good/died 7 months after op due to panleukopenia
4	BSH, intact female, 1 y, 1.7 kg	Trauma; fall from a height	TMH ankylosis, mandibular deviation	Bilateral extra-intra ankylosis, asymmetric malocclusion	Bilateral	Excellent/11 m
5	DSH, intact male, 8 m, 1.5 kg	Trauma; traffic accident	TMJ ankylosis, dehydration	Left intra ankylosis	Unilateral	Good/19 m
6	SF, neutred female, 1 y 5 m, 1.8 kg	Trauma; fall from a height	TMJ ankylosis	Left extra-intra ankylosis	Unilateral	Fair/20 m
7	SF, intact female, 5.5 m, 1 kg	Trauma; fall from a height	TMJ ankylosis, mandibular asymmetry	Left extra-intra ankylosis, asymmetric malocclusion	Bilateral/ in different sessions	Poor after 1st op, excellent after 2nd op/33 m
8	DSH, neutred female, 6y, 3.2 kg	Trauma; traffic accident	TMJ ankylosis, gingivitis, left sided facial paralyses	Right intra ankylosis	Unilateral	Good/30 m
9	SF, Female, intact, 4 m, 1 kg	Trauma; fall from a height	TMJ ankylosis, mandibular brachygnathia	Left intra ankylosis, malocclusion	Bilateral/ in different sessions	Poor after 1st op, excellent after 2nd op/7 m
10	DSH, neutred male, 3 y 7 m, 2.8 kg	Trauma; fall from a height	TMJ ankylosis	Left intra ankylosis	Unilateral	Good/9 m

BLH: British Longhair, BSH: British Shorthair, DSH: Domestic Short Hair, m: Months, op: Operation, SF: Scottish Fold, TMJ: Temporomandibular Joint, y: Year(s)

them more susceptible to ankylosis [6,10]. An interesting aspect of this study was the predominance of purebred cats including British Shorthair and Scottish Folds. Breed predisposition has not been previously reported in the veterinary literature; however, the relatively high representation of these breeds in our series may suggest anatomical or behavioral factors that could increase susceptibility to maxillofacial trauma or post-traumatic ankylosis. Although further studies with larger and genetically diverse populations are required to confirm any potential breed predisposition, these findings raise awareness of the need for vigilance in these breeds following facial trauma.

Another notable observation was that most owners recognized early signs such as difficulty eating or decreased grooming but only sought veterinary care when the mouth could no longer be opened. This delay in presentation may contribute to the progression from fibrous to bony ankylosis, as previously suggested [6,8,18]. Early recognition and imaging are therefore critical to prevent complete ossification and improve surgical outcomes.

In six cats, there was a history of prior attempted jaw manipulation under general anesthesia at other veterinary centers. These procedures involved forceful opening of the mouth to restore motion, but only transient improvement was achieved, and all cats later presented with recurrence and more severe restriction. Similar cases have been reported by Maas and Theyse [5], who warned that forceful manipulation of ankylosed jaw joints may exacerbate intra-articular trauma and fibrosis, thereby worsening the condition. Later studies have also reported recurrence after unsuccessful conservative interventions [11,18]. The current findings support these concerns and highlight the need for accurate imaging and appropriate surgical planning rather than non-specific manual interventions.

Unilateral involvement was more common than bilateral, as also observed in other studies [10,11]. However, in the present series, nearly all unilateral cases affected the left TMJ, a distribution not previously emphasized in the literature. This may be related to the direction of traumatic forces, or it may be due to random variation within the small cohort. Further studies involving larger populations are needed to determine whether there is a biomechanical or behavioral basis for laterality in feline TMJ pathology.

Gap arthroplasty restored immediate postoperative mouth opening in all cats, which aligns with previous studies showing rapid functional recovery after removal of ankylotic bone bridges and degenerated joint [10,11,18]. Ankylosis recurred in two cats, and clinical examination determined that it most likely developed on the contralateral side. Clinical signs completely resolved following contralateral surgical intervention. This finding reflects observations in other reports suggesting that bilateral involvement may initially be subclinical and subsequently manifest as contralateral ankylosis [18]. These findings emphasize the importance of detailed preoperative CT evaluation to detect even the slightest changes in the contralateral joint.

The use of piezoelectric bone cutting devices in feline TMJ arthroplasty allows for precise osteotomy near critical neurovascular structures [11,25]. Although previous studies have emphasized the accuracy and improved visibility of these devices [26], traditional instruments such as osteotomes, mallets and rongeurs were successfully used in this study. While piezo surgery provides minimally traumatic cutting, it requires specialized equipment that is not always available in clinical settings. The positive outcomes in this series demonstrate that traditional instruments remain a practical and cost-effective alternative when careful technique and visualization are ensured.

Postoperative physiotherapy and early functional use of the mandible are crucial for functional recovery following temporomandibular joint surgery in cats, in addition to preventing fibrosis and recurrence [5,11,14,24]. In the current study, owners were instructed to perform gentle stretching and encourage chewing activity using toys or chewing treats. Although adherence was not objectively monitored, the low recurrence rate suggests that even simple, consistent exercises can contribute to favorable outcomes.

Although gap arthroplasty remains the most widely described surgical technique for the management of feline TMJ ankylosis, other procedures such as interpositional arthroplasty (IA) and segmental mandibulectomy (SM) have been proposed to reduce the likelihood of recurrence. In a retrospective study of Kocsis et al. [18] found that interpositional arthroplasty (IA) was advantageous compared with gap arthroplasty (GA) and segmental mandibulectomy (SM). The use of autologous fat grafts in interpositional arthroplasty (IA) has attracted attention due to their excellent biocompatibility and cushioning effects, and they function as biological spacers to reduce the risk of re-ankylosis. However, long-term outcomes remain uncertain due to potential fat resorption and fibrosis. As in this study, fat harvesting may be difficult in cats with poor body condition, and additional manipulation may increase surgical stress and the risk of infection. IA also prolongs the duration of surgery and increases the risk of postoperative inflammation compared to GA. Therefore, while GA remains the most practical option for advanced cases, IA may be considered for recurrent cases once the general condition and body weight have improved.

Complications were rare in the present series. Only one cat developed postoperative mild dermatitis due to early removal of the Elizabethan collar, which was resolved with therapeutic laser sessions. No major perioperative or postoperative complications such as hemorrhage, infection, or facial nerve damage occurred. Although two cases required contralateral surgery, this was not considered a postoperative complication. It was presumed to result from subtle pre-existing changes in the opposite joint that were not detectable on the initial CT scan. This compares favorably with previous reports, in which postoperative complications such as mandibular deviation, malocclusion, or fibrotic relapse were occasionally observed [5,11]. The limited lateral mandibular movement characteristic of feline TMJ anatomy may reduce the risk of postoperative asymmetry.

Long-term follow-up (mean = 16.8 months) confirmed sustained functional improvement in all surviving cats. Owner-reported quality of life was markedly improved in nearly all cases, consistent with the functional classifications proposed by Aghashani et al. [11] and Kocsis et al. [18]. Only one owner reported moderate improvement, with the cat continuing to eat soft food. These subjective findings provide valuable complementary data to clinical assessment, reflecting the true impact of surgery on daily behavior and welfare.

The main limitations of this study include the absence of quantitative measurement of mouth opening, the retrospective design, and the limited number of cases. Nevertheless, the consistency of improvement across cases, the relatively long follow-up period, and the inclusion of different breeds strengthen the reliability of the findings. Overall, this study supports GA as an effective, accessible, and low-risk procedure for restoring jaw function in cats with TMJ ankylosis.

Another limitation of the present study was the absence of postoperative imaging, primarily due to the need for general anesthesia and the associated financial constraints faced by most owners. Although postoperative computed tomography would have provided objective confirmation of the surgical gap and assessment of bone remodeling, it was not feasible under these conditions. Follow-up radiographs were also considered; however, deep sedation or anesthesia would still have been required to obtain diagnostic-quality images. Also, most of the owners were unwilling to admit their cats once the favorable

improvement was achieved. Consequently, postoperative evaluation relied mainly on clinical examination and owner-reported functional improvement. Despite this limitation, the consistent recovery of mandibular motion and the absence of clinical recurrence in most cases suggest that the outcomes were accurately reflected by the functional assessments performed.

In conclusion, gap arthroplasty has effectively restored mandibular function and improved quality of life in cats with TMJ ankylosis. When meticulous dissection and visualization are ensured, this procedure can be performed safely and successfully using conventional instruments. The findings emphasize the importance of early diagnosis and appropriate referral, particularly in cases involving delayed presentation or prior unsuccessful interventions. Long-term outcomes were favorable, with recurrence limited to cases requiring contralateral surgery. GA remains a practical and reliable treatment option for TMJ ankylosis in cats.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (P. Can).

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Conflict of Interest: The authors declare that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Authors' Contributions: PC designed the study, examined the cases, evaluated the CT images, performed the surgeries, reviewed the patients' database and wrote the original draft. EU reviewed the patients' database, examined the cases, and performed the surgeries. RE examined the cases, assisted the surgeries, collected the follow-up data. All authors reviewed and approved the final version of the manuscript.

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LETTER TO THE EDITOR

A Note on the Co-Infection Dynamics of Lumpy Skin Disease (LSD) and Bovine Haemoprotozoan Parasites

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Dear Editor,

Arthropod-borne diseases pose a serious threat to livestock health and significantly impact the economic stability of the livestock sector. Among these, Lumpy Skin Disease (LSD), caused by the Lumpy Skin Disease Virus (LSDV) from the family Poxviridae, is a significant concern. Primarily affecting cattle, LSD is widespread in Asia and results in substantial economic losses. The disease is mainly transmitted by hematophagous vectors like stable flies -*Stomoxys* spp., *Haematobia* spp.[1], mosquitoes [2], and ticks- particularly those of the *Hyalomma* spp.[3] and *Riphicephalus* spp. [1,4]. Likewise, ticks serve as vectors for several hemoprotozoan pathogens such as *Theileria* spp., *Babesia* spp., and *Anaplasma* spp., which cause theileriosis, babesiosis, and anaplasmosis, respectively. In recent years, reports of co-infections involving LSD and hemoprotozoan parasites from endemic areas have increased. The overlapping ecology and shared tick vector populations facilitate the simultaneous transmission of LSDV and hemoprotozoa. These co-infections exacerbate clinical signs, delay recovery, and complicate diagnosis, leading to additional economic losses in affected herds.

Previous research has shown that LSD often coexists with haemoprotozoan infections such as babesiosis [1] , theileriosis [1,4], and anaplasmosis [4]. Histopathological links between LSD and theileriosis have also been observed [5]. LSDV DNA in tick salivary glands reinforces the idea that ticks play a role in transmitting the virus [4]. The involvement of *Hyalomma anatolicum anatolicum* ticks in spreading LSD, theileriosis, and anaplasmosis is well established [4]. Likewise, the association of *Riphicephalus* spp. ticks, that is a common vector for bovine babesiosis, also spread LSD [1,4].

Breed susceptibility also plays a key role. Holstein Friesian cattle have been reported to develop more severe forms of LSD compared to indigenous breeds [6]. Similarly, exotic breeds are more prone to haemoprotozoan infections such as theileriosis. The higher prevalence of coinfections can be linked to the common vector -the tick- which transmits LSDV and haemoprotozoa [3]. Abas et al.[7] found a strong correlation between LSD outbreaks and haemoprotozoan infections, showing a significant difference ($P<0.05$) in parasitemia levels between LSD-positive and LSD-negative cattle. This difference was due to the immunosuppressive effect of LSDV.

In haemoprotozoan infections, parasitaemia levels are closely linked to the phagocytic activity of leukocytes, which becomes significantly impaired during LSD infection. Typically, parasite invasion triggers an innate immune response through chemokine release and recruitment of phagocytic cells [8-10]. This defence mechanism is particularly effective during acute infections with high parasitaemia, helping to control the infection [9]. However, during LSD infection, this immune response is disrupted, resulting in altered parasitaemia levels and more severe disease progression. Additionally, animals with tropical theileriosis experience dysfunction in key immune cells -macrophages, neutrophils, B cells, and T lymphocytes (CD4+ and CD8+)- which collectively maintain immune balance [11,12]. Any disturbance in their activity predisposes

animals to secondary viral infections, including LSDV [5]. Consequently, once coinfection occurs, both diseases worsen due to the host's weakened immune system.

Future investigations should focus on molecular and immunological methods to better understand the dynamics of these concurrent infections. The immunosuppressive effect of LSDV can make animals more vulnerable to secondary haemoprotozoan infections or trigger latent parasitic infections. Conversely, previous haemoprotozoan infections may weaken immune function, increasing susceptibility to LSDV. Real-time field and experimental studies are necessary to determine whether LSD predisposes animals to haemoprotozoan infections or the other way around. The possibility that carrier or sub clinically infected animals may become more susceptible to LSD also needs further study.

From a diagnostic standpoint, coinfection can mask typical clinical signs, resulting in underdiagnosis or misdiagnosis. Thus, a thorough diagnostic strategy that includes clinical evaluation, blood smear analysis, and molecular testing is vital for precise identification. Prompt diagnosis and immediate treatment are key to lowering morbidity, avoiding economic costs, and enhancing recovery in cases of coinfection.

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31th Volume Index

Author Index

31th Volume Index

Author Index

31th Volume Index

Author Index

31th Volume Index

Author Index

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's publication policy and ethical principles include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

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The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

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Volume: 31, Issue: 6, Year: 2025

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• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

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Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

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Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

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The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

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Kafkas Univ Vet Fak Derg
Volume: 31, Issue: 6, Year: 2025

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Kafkas Univ Vet Fak Derg
Volume: 31, Issue: 6, Year: 2025

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Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

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Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

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7- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

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References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

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DOI number should be added to the end of the reference.

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