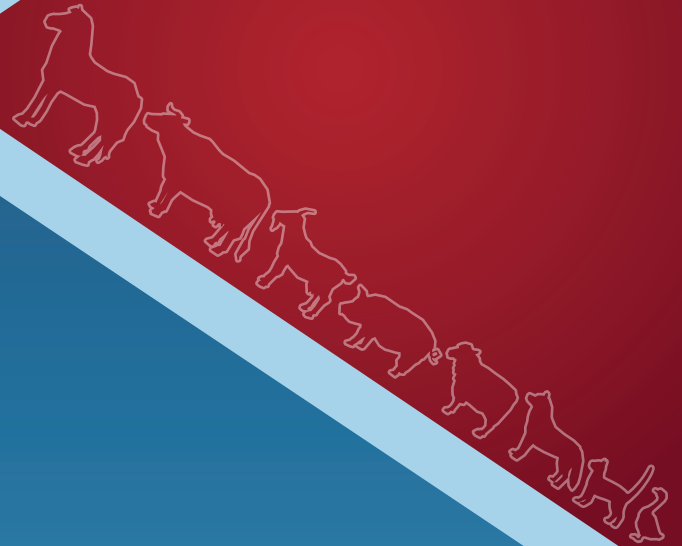


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REVIEW ARTICLE

Perioperative Nociception and Pain in Dogs and Cats - Part I: An Illustrative Text on Pain Mechanisms, Models and Assessment

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Abstract

Effective perioperative pain management depends on a thorough understanding of pain mechanisms and reliable assessment methods. Pain serves as a vital protective mechanism, acting as an alarm for underlying disease, trauma, or surgical injury. In disease, it prompts investigation of causative factors, while in trauma or surgery, it encourages protection of injured tissues. Inadequate management of acute pain can lead to significant physiological and behavioral consequences, including stress hormone release, increased heart rate and blood pressure, behavioral alterations, delayed wound healing, chronic pain development, and prolonged hospitalization. The purpose of this review is to enhance clinicians' and researchers' understanding of acute pain and nociception in dogs and cats, with particular emphasis on perioperative settings. The physiological processes involved in pain transmission, modulation, perception, as well as peripheral and central sensitization are discussed. Additionally, commonly used subjective and objective pain assessment techniques, including behavioral scoring systems and physiological indicators, are reviewed to support accurate pain recognition. This review is presented in two complementary parts. Part I focuses on pain pathophysiology and assessment, forming the foundation for Part II, which evaluates pharmacological and multimodal analgesic strategies for perioperative pain management.

Keywords: Cats, Dogs, Nociceptive pathways, Pain assessment, Perioperative pain, Sensitization

INTRODUCTION

Pain functions as a natural alarm to an underlying disease, trauma or surgery. In disease, it prompts investigation of the cause(s), whereas in trauma or surgery, it acts as a protective warning to safeguard the injured site against further damage. Absence or inadequate perception of pain may result in undetected disease progression and insufficient protection of injured tissues, ultimately leading to adverse outcomes. Poorly managed acute

pain can trigger stress hormone release and is associated with reduced activity, increased heart rate and blood pressure, behavioral alterations, shivering, hypersensitivity, aggression, vocalization, anorexia, weight loss, delayed wound healing, depression, transition to chronic pain, and prolonged hospitalization. Therefore, this review aims to discuss the pathophysiology, recognition, assessment, and management of acute pain in veterinary patients, with emphasis on improving animal welfare and clinical outcomes.



DEFINITION: PAIN AND NOCICEPTION

The official definition of pain by the International Association for the Study of Pain (IASP) is: "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" ^[1,2].

Clinically, pain can be classified as either acute or chronic. Acute pain results from an injury and ends with wound healing. Chronic pain lasts beyond acute pain or wound healing.

Generally, pain that persists for more than three months is considered chronic pain. This suggests that there is an ongoing slow change over time in the mechanism leading to chronic pain; and that there is not anything that otherwise causes "chronic pain" after three months. Typical example of causes of pain is surgical pain, trauma, or onset of acute disease, such as pancreatitis, whereas osteoarthritis, cancer and periodontal disease are examples of conditions in which chronic pain is seen. Surgical pain that exists beyond the expected time frame of acute pain is called chronic pain ^[1,3,4]. In animals, verbal communication is not comprehensible across different species, hence, making the task of pain assessment more challenging for clinicians and researchers ^[5]. It is highly important for a clinician to have a good understanding of pain physiology, pathways, and assessment methodology in order to treat it effectively.

Let us briefly consider why animals do not consciously perceive pain under general anesthesia. If pain perception is absent, one may question why physiological parameters such as heart rate, blood pressure, and respiratory rate increase in response to surgical stimulation during anesthesia, while animals may vocalize due to pain immediately upon recovery.

This phenomenon is explained by the process of nociception, which refers to the neural encoding and processing of noxious stimuli ^[6,7]. Nociception is distinct from pain in that it does not involve a conscious emotional experience; rather, pain requires processing at the level of the conscious brain. Under general anesthesia, cortical function and the emotional component of pain perception are suppressed. However, nociceptive pathways at the peripheral and spinal level may remain active.

As a result, surgical stimuli can continue to generate nociceptive input, leading to autonomic responses such as increases in heart rate, blood pressure, and respiratory rate, despite the absence of conscious pain perception. Importantly, once the animal recovers from anesthesia and cortical function is restored, this nociceptive input can then be perceived as pain, resulting in behaviors such as vocalization.

Nociception begins at the site of tissue injury, where specialized receptors termed nociceptors transduce noxious stimuli into electrical signals. These signals are transmitted through the nervous system for further processing. Thus, while nociception can occur in the absence of conscious pain, the perception of pain requires intact central processing and does not occur without nociceptive signaling.

Nociceptors

The receptors characterised by free, non-encapsulated nerve endings that detect nociceptive (noxious) stimuli are called nociceptors. These are widely distributed in the skin and deep tissue, with their cell bodies located in dorsal root and trigeminal ganglia ^[1]. These nociceptors are peripheral terminals of primary afferent neurons. Mechanical, chemical and thermal stimuli can stimulate these receptors. Some nociceptors respond to only one of these stimuli (unimodal), whereas others respond to a variety of stimuli (polymodal) ^[6,7].

Sensory Nerve Fibers: Three classes of sensory nerve fibers have been described based on anatomy (myelination), function and speed of conduction. Large, myelinated group A α and A β nerve fibers - these are low-threshold fibers with a diameter of 10 μ m and detect low-intensity, innocuous (touch, vibration, pressure, and limb movement) stimuli with high velocities of 30 to 100 m/s. Smaller, lightly myelinated A δ fibers and nonmyelinated and nonmyelinated C fibers - A δ and C fibers are high threshold fibers and are more predominant (>90%) than low threshold fibers ^[8]. A δ fibers conduct at a speed of 5 to 30 m/s and detect sharp pricking pain and result in rapid withdrawal from the stimulus as a primary warning (fast pain). C fibers conduct at speeds of 0.5 to 2.0 m/s and detect slow burning pain (slow pain) and are primarily involved in nociceptive signalling. Both A δ and C fibers supply to the skin and deep somatic or visceral structures ^[1]. Silent nociceptors represent a subset of C-fiber afferents characterized by a high activation threshold and minimal or absent responsiveness to mechanical or thermal stimuli under normal physiological conditions. Exposure to inflammatory mediators significantly decreases their threshold, and these previously silent nociceptors become stimulated and sensitive to a variety of stimuli ^[1,9].

Activation of pain receptors in the peripheral tissue (skin) generates action potentials that are conveyed to the central nervous system (CNS) through A δ and C nerve fibers. Sharp, pin prick and injurious pain (first pain) are transmitted by fast A δ fibers. However, dull aching pain (second pain) is transmitted by slow C fibers. The primary sensory neurons enter the spinal cord and synapse with neurons in the dorsal horn. From the dorsal horn, second-order neurons having long axons cross in the

anterior commissure and pass through the spinothalamic tract towards the brain. Some long axons that synapse with C neurons do not cross but pass cranially in the ipsilateral anterolateral spinal pathway. Fibers from the anterolateral spinal pathway terminate in the thalamus from where signals are passed to the sensory cortex and other centers in the CNS, where pain perception takes place. Pain consists of three main components: a sensory-discriminatory component (temporal, spatial, thermal/mechanical), an affective component (subjective and emotional, describing associated fear, tension, and autonomic responses) and an evaluative component, describing the magnitude of the quality (e.g. stabbing/pounding; mild/severe) [1,10-12].

ANATOMY OF THE SPINAL CORD

Fig. 1 illustrates the anatomy of the spinal cord which is classified into gray and white matter. In a broader overview, the gray matter is divided into three distinct regions: dorsal horn, intermediate zone, and ventral horn, which are further subdivided into ten undemarcated laminae (Fig. 1). The dorsal horn comprises laminae I to VI, the intermediate zone comprises lamina VII, the ventral horn comprises laminae VIII to IX, and lamina X surrounds the central canal of the spinal cord [13]. In primary afferent fibers, the thinly myelinated A δ fibers project to lamina I and V, thick myelinated A β fibers project to lamina III, VI and V, while, non-myelinated C fibers project to lamina I and II in the dorsal horn of the spinal cord.

Electrophysiological studies have revealed that spinal cord neurons within lamina I generally respond to noxious stimuli, neurons in laminae III and VI respond to non-noxious stimuli, and neurons in lamina V receive both noxious and non-noxious information. Neurons in lamina V are called wide dynamic range neurons (WDR) given their response to a range of intensities from non-noxious to noxious stimuli [14]. Neurons in lamina I and

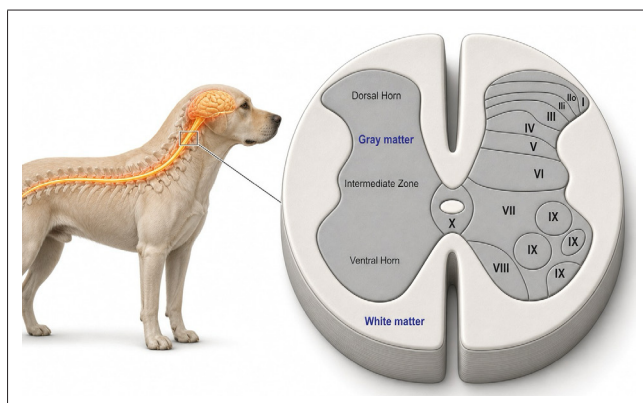


Fig 1. Anatomy of the spinal cord. Classified into gray and white matter. The gray matter is divided into three distinct regions: dorsal horn, intermediate zone, and ventral horn, which are further subdivided into ten undemarcated laminae

V carry nociceptive information to the thalamus and brainstem via spinothalamic and spinoreticulothalamic tracts. The spinothalamic tract is relevant to the sensory-discriminative aspect of the pain experience, whereas the spinoreticulothalamic tract is more relevant to poorly localized pain. From the brain stem and thalamus, the information reaches the cortical structures [14].

NOCICEPTIVE PATHWAY

Nociceptive pathways comprise of ascending and descending pathways. Propagation of noxious information from the site of injury towards the brain occurs through the ascending pathway, whereas movement of information from the brain to the periphery occurs through the descending pathway. This processing of information occurs through the phenomenon of nociception.

Nociception

Nociception is a phenomenon by which intense mechanical, thermal or chemical noxious stimuli are detected by nociceptors, a subpopulation of peripheral nerve fibers [14]. The process of nociception takes place in a few steps: transduction, transmission, modulation projection and perception (Fig. 2, Fig. 3).

When tissue is damaged, noxious stimuli are translated into nociceptive signals through a process called transduction. Transmission is the process by which these signals from the periphery reach the spinal cord. Along the nociceptive pathway, signals undergo both inhibitory

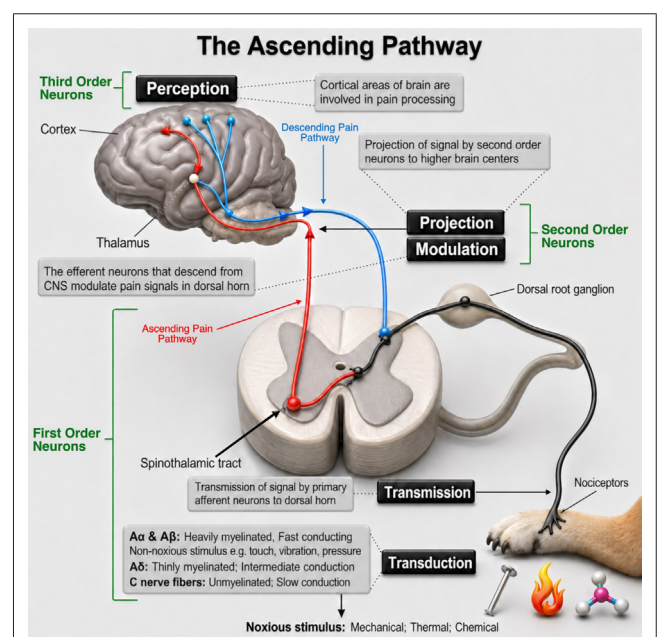


Fig 2. Illustrative summary of the ascending pathway of nociception. When tissue is damaged, noxious stimuli are translated into nociceptive signals through a process called transduction followed by transmission, modulation, projection and perception. Phases of the pain pathway, types of nerves involved, and anatomical regions have been labelled

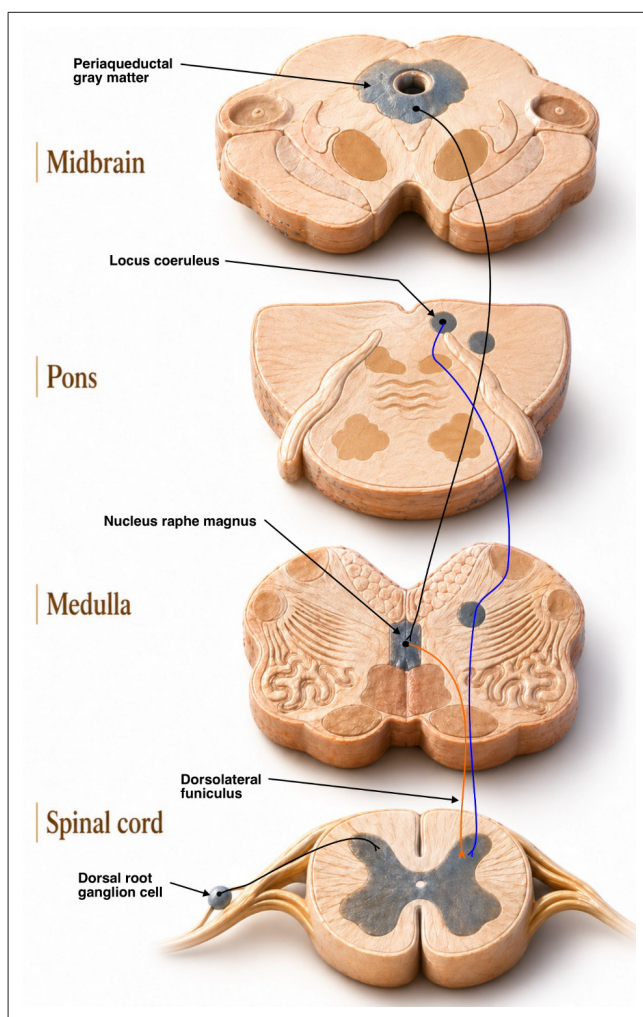


Fig 3. The descending inhibitory pathway. Periaqueductal gray matter (PAG) and rostroventral medulla (RVM) are the main regions involved in the descending inhibitory pathway. Both centers have high concentrations of opioid receptors and endogenous opioids. The PAG receives inputs from brain regions of interest, such as the thalamus, hypothalamus and reticular formation, and sends to the nucleus raphe magnus and locus coeruleus. These centers project to the dorsal horn to inhibit pain transmission

and excitatory processes, collectively termed modulation. This process regulates the intensity and transmission of nociceptive input through mechanisms within the peripheral nervous system, spinal cord, and brain.

Projection refers to the ascending transmission of nociceptive signals from the spinal cord to higher brain centers. This process ultimately leads to perception, the final stage of nociception, in which these signals are consciously interpreted as pain [15].

Transduction: When nociceptors are stimulated by noxious stimuli (heat, cold, chemical or mechanical), this results in a conformational change in the receptor. With this change in the receptor the noxious signals are converted into electric current by activating the opening of depolarizing cationic ion channels or the closing of outward potassium channels [16]. Various molecules, such

as members of the transient receptor potential (TRP) cation channel family, acid-sensitive ion channels (ASIC) and purine-activated ligand-operated ion channels, have been identified in the process of transduction. The TRP family members include, transient receptor potential subfamily ankyrin (TRPA), transient receptor potential subfamily canonical (TRPC), transient receptor potential subfamily melastatin (TRPM), and transient receptor potential subfamily vanilloid (TRPV) [17,18] transduce thermal and chemical signals. Certain receptors of the TRP family are activated by a decrease in local pH, or chemical ligand, i.e., capsaicin (the vanilloid constituent that provides the “hot” in hot peppers). The TRPA and TRPM share the role of transducing thermal signals with many two-pore potassium channels (K2P) and Na_v1.8 channel [19]. Ischemia and inflammation result in an increase in hydrogen ion concentration, which activates ASIC. Damage to cells leads to excesses of adenosine triphosphate (ATP) that signal to purinergic receptors; P2X is a purine-activated ligand-operated ion channel, and P2Y is a G-protein-coupled receptor. Bacterial lipopolysaccharides and inflammatory cytokines result in expression of the bradykinin-1 (B1) receptor, while the bradykinin-2 (B2) receptor is constitutively expressed. Activation of B1 and B2 forms inositol 1,4,5-triphosphate and diacylglycerol with subsequent increases in intracellular calcium concentration. Activation of bradykinin B₂ receptors leads to the production of prostaglandin E₂ (PGE₂) and prostacyclin (PGI₂), which act to sensitize nociceptors and amplify the nociceptive signal [20]. Piezo channels, TRPA1 and TRPV4, transient Ca²⁺ channels, and at least three K2P channels are among the transducers known to be involved in mammalian mechanotransduction [21-26]. Clinically, activation of these receptors leads to heat hypersensitivity as a result of tissue injury and inflammation, contributing significantly to the development of painful condition.

Recent research has revealed that TRPV1-4 channels play a role in transduction of chemical and thermal stimuli and are expressed in afferent nociceptors, and pain-sensing neurons [27]. Two of the channels, TRPA1 and TRPM8, are activated by cold temperature [28]. These channels have been proposed as a therapeutic target due to their association with heat and cold thermal stimuli. Further research into nociception will facilitate the development of more effective and targeted therapeutic strategies for the management of pain and its associated pathologies.

Transmission: Once the successful transduction of nociceptive stimuli by primary afferent neurons takes place, different voltage-gated ion channels are activated by receptor potentials. Voltage-gated sodium and potassium channels play significant roles in the generation of action potentials, which transmit nociceptive signals to the dorsal horn [14]. Tetrodotoxin (TTX)-resistant channels,

TTX-sensitive channels and voltage-gated sodium channels (Na_v) 1.1, 1.6, 1.7, 1.8 and 1.9 are different types of voltage-gated sodium channels [29]. Tetrodotoxin (TTX)-sensitive channels cause rapid depolarisation and are responsible for acute pain signal transmission. Tetrodotoxin (TTX)-resistant channels are responsible for transmitting slow, burning, or inflammatory pain signals (neuropathic pain). Clinically these receptors are related to several chronic pain conditions, manifested as hyperalgesia and allodynia. When the threshold potential is reached, an action potential is generated, allowing propagation of the nociceptive signal along the neuron. This action potential arises through the coordinated opening of voltage-gated sodium (Na^+) and calcium (Ca^{2+}) channels, which mediate membrane depolarization, along with the relative reduction in potassium (K^+) efflux that otherwise contributes to membrane hyperpolarization [20]. Nociceptive signals are transmitted to the CNS via the dorsal horn of the spinal cord. The cell bodies of primary afferent nociceptors are located in the dorsal root ganglia. Within the dorsal horn, primary afferent neurons synapse with secondary neurons and interneurons across different laminae. These connections play a critical role in the integration, modulation, and further transmission of nociceptive information to higher centers of the brain.

Modulation or Descending Inhibitory Pathway: The pain signals in the dorsal horn are modulated by efferent neurons that descend from the CNS. In addition to antinociceptive pathways, descending pathways exist with the ability to modify the pain reaction. The descending pathways include thalamocortical structures, pons of the brain stem, the PAG of the midbrain, the rostroventral medulla (RVM), and the dorsal horn; PAG and RVM are generally considered core regions of this important system (Fig. 3) [30,31].

Modulation of plasticity augments sensitivity (excitatory) to incoming nociceptive signals; this modulation takes place after release of excitatory neurotransmitters such as substance P, glutamate, and calcitonin gene-related peptide (CGRP). Augmented sensitivity is facilitated by upregulation of excitatory neurotransmitter receptors (Structural Plasticity) and decreased signal intensity required to induce conformational changes in receptor proteins (functional plasticity). On the other hand, sensitivity of secondary neurons to incoming information is decreased due to hyperpolarization in the postsynaptic nerve terminals or decreased influx of calcium into presynaptic terminals. This decrease in sensitivity (inhibitory) occurs after the release of GABA, endorphins and enkephalins, norepinephrine, and serotonin acting at some serotonin receptors [30,31].

“On” and “Off” cells are the unique populations within the

RVM. Off-cells hyperpolarize in response to activation of the spinothalamic tract and decrease the transmission of the streams of nociceptive action potentials in the brain stem [32]. On the other hand, nociceptive information from the spinothalamic tract causes excitation of on-cells and engages parabrachial, hypothalamic, cingulate, insular, and septohippocampal pathways subserving arousal and aversive reactions to pain [33]. These on-cells appear to be important in producing hyperalgesia by maintaining central sensitization after peripheral tissue injury [32,34]. This understanding of on-cells and central sensitization is crucial for managing chronic pain conditions, where hyperalgesia can significantly impact a patient's quality of life.

Projection and Perception: The dorsal horn of the spinal cord possesses bodies of second-order nociceptive neurons. From the dorsal horn, these second-order neurons carry nociceptive impulses through their axons projecting to higher CNS centers, which are responsible for the processing of nociceptive information. The cortical regions involved in the pain processing are the primary and secondary somatosensory cortex and its vicinity in the parietal operculum, insula, anterior cingulate cortex and prefrontal cortex. These are collectively called pain matrices [35]. Imaging studies have revealed that the thalamus, insula, hypothalamus and ventral tegmental area (VTA) are associated with the stimulation of C fibers in response to noxious stimulation in rats [36].

The wide dynamic range and high threshold neurons (spinothalamic tract) pass through the pons, medulla and mid-brain to terminate in specific portions of the thalamus. The somatosensory cortex receives nociceptive signals from the thalamus. The reticular formation also receives collateral branches from the spinothalamic tract. Emotional exhibition of pain and sensory discrimination takes place when the signals pass through these tracts. Emotional components of pain, including somatic and autonomic motor reflexes as well as increased arousal is probably the function of the reticular formation [37,38].

Peripheral nociceptors ($\text{A}\delta$ and C fibers), responsible for transduction and transmission are slowed down or blocked by anaesthetics at the periphery. Dorsal root ganglia serve a role of relay station for nociceptive information to the CNS, which is the area of modulation of the nociceptive information. General anaesthetics suppress transmission of nociceptive signals within ascending pathways, including the spinothalamic tract, thereby preventing conscious perception of pain. The brain stem regulates respiration and CVS; anaesthetics have minimal depression effects on these centers compared to the thalamus and cortex, responsible for nociception and pain [39-42].

MECHANISMS OF SENSITIZATION

Sensitization is defined by the International Association for the Study of Pain (IASP) as the 'Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs. Peripheral sensitization refers to an increased responsiveness in the nociceptive neurons in the periphery, whereas central sensitization refers to an increased responsiveness of nociceptive neurons in the central nervous system (CNS) to their normal or subthreshold afferent input' [43,44].

Hyperalgesia and allodynia are typical clinical characteristics of sensitization. Hyperalgesia is an exaggerated and prolonged response to a noxious stimulus, while allodynia is a pain response to a low-intensity, normally innocuous stimulus such as light touch to the skin or gentle pressure. Hyperalgesia and allodynia are consequences of peripheral and central sensitization [1].

Mechanism of Peripheral Sensitization

Tissue damage during surgery results in the accumulation of endogenous factors produced from stimulated nociceptors and non-neural cells that reside within or

infiltrate the injured area. These non-neural cells include mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts. Neuropeptide substances such as substance P, nerve growth factor (NGF) and calcitonin gene-related peptide (CGRP) are produced after the noxious stimulus has been recognized by the sensory nerve endings of peptidergic nociceptors. The release of substance P and CGRP induces vasodilatation, plasma extravasation, and other effects, thus producing 'neurogenic inflammation' [45]. Discharge of vasoactive substances from mast cells and increased vascular permeability leads to neurogenic edema [46].

Histamine and serotonin are also released as a result of increased permeability and act as algogenic substances. Substance P, NGF, kinin and interleukin-I cause degranulation of mast cells, releasing histamine, which acts on sensory neurons to produce pain and itching [47]. This activation of sensory neurons by histamine induces the discharge of neuropeptides and prostaglandins (PGs), resulting in aggravation of inflammation and hyperalgesia. Serotonin released from platelets and mast cells during injury is a major inflammatory mediator during the initial phase of inflammation. Serotonin directly stimulates sensory neurons via activation of the 5HT type-3 (5HT₃) receptor. Norepinephrine is released as a result of stimulation of the sympathetic nervous system in response to nociceptive impulses. Norepinephrine accelerates sensitization of the nociceptors, creating another vicious cycle [48]. Fig. 4 illustrates the vicious and complex mechanism of peripheral sensitization.

In summary, these mediators augment the excitability of nerve fibers, resulting in a decreased activation threshold of nociceptors and, consequently, amplifying the sensitivity to thermal and mechanical stimuli in the surrounding injured tissue. Subsequently, silent nociceptors are also recruited and activated [16]. In addition to neurotrophin injury, these mediators also cause the release of cytokines; among them, interleukin- β (IL- β), IL-6 and tumor necrosis factor α (TNF- α) are the key players in nociception. These cytokines act on nociceptors and contribute to pain hypersensitivity by increasing the production of PGs, bradykinin and extracellular protons [14]. This cascade of events produces an "inflammatory soup" [49]. This inflammatory soup bathes peripheral nociceptors, consequently decreasing their activation threshold or augmenting their sensitivity to pain, resulting in peripheral sensitization [50].

Peripheral sensitization is characterized by allodynia and hyperalgesia. In allodynia, activation thresholds are decreased, resulting in pain from the stimulus that does not normally provoke pain. In hyperalgesia, there is increased pain from the stimulus that normally provokes pain [2]. This hyperalgesia at the site of injury is called

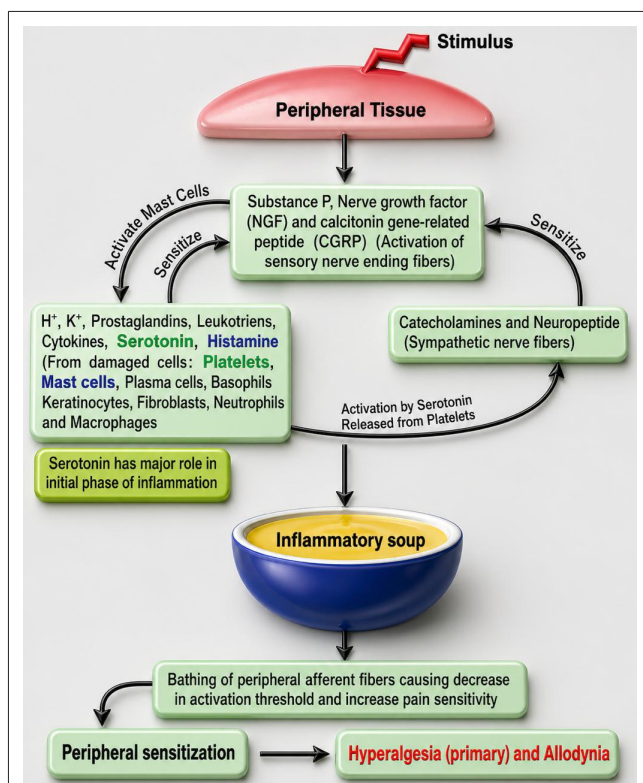


Fig 4. Illustrative summary of peripheral sensitization, where a vicious cycle of neural and non-neural mediators created an inflammatory soup to develop peripheral sensitization. This cascade of events produces an "inflammatory soup", which bathes peripheral nociceptors, consequently decreasing their activation threshold or augmenting their sensitivity to pain, resulting in peripheral sensitization

primary hyperalgesia, which is commonly observed following surgery and other types of trauma. However, hypersensitivity at the site of uninjured skin around the wound is called secondary hyperalgesia, which is the function of central sensitization^[51]. Activation of silent nociceptors results in mechanical hypersensitivity^[10], which is an important feature of inflammatory pain and a result of central sensitization^[49].

Mechanism of Central Sensitization

Central sensitization takes place at the level of the dorsal horn. When the noxious stimulus is encoded into an electrical signal, these signals are then transmitted to secondary neurons in the dorsal horn of the spinal cord from the site of injury by primary afferent nerve fibers. The secondary neurons are divided into two classes, "nociceptive specific" or "high threshold" and "wide dynamic range" or "convergent". Nociceptive-specific neurons respond exclusively to noxious stimuli from C and A δ fibers. Wide dynamic range neurons respond to both noxious and non-noxious stimuli. Bombardment of WDR and nociceptive-specific neurons by afferent C fibers in the dorsal horn of the spinal cord results in a progressive increase in the frequency of firing of second-order spinal neurons at a frequency above 0.5 Hz. This is also known as windup, in which magnesium blocking of the N-methyl-D-aspartate (NMDA) receptors

is removed, rendering them for glutamate activation^[20].

Glutamate is the main and fast neurotransmitter in the CNS and plays a major role in nociceptive transmission. Glutamate acts at α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, NMDA receptors, kainate (KA) receptors, and metabotropic glutamate receptors. Glutamate-activated NMDA receptors are involved in the mechanisms that result in increased synaptic efficiency. Phosphorylation of NMDA receptor activates its mobility from intracellular store to synaptic membrane, enhancing its responsiveness to glutamate by removal of the voltage-dependent magnesium (Mg^{++}) ion block, thereby favoring the open-state configuration (Fig. 5)^[52]. Glutamate brain-derived neurotrophic factor (BDNF) and substance-P activate membrane-bound receptors (NMDA, metabotropic glutamate receptor, neurokinin receptor and tropomyosin receptor kinase B), resulting in increased intracellular calcium and activation of intracellular molecular signaling cascades, including calcium-calmodulin-dependent kinase [CaMK], protein kinase C, protein kinase A, neuronal cyclooxygenase (COX), nitrous oxide synthase and activation of other calcium-sensitive transcription factors. Consequently, this cascade assists in further receptor function and expression of cell surface receptors^[20].

Phosphorylation of ion channels and membrane receptors are the post-translational changes caused by activation of the gamma isoform of protein kinase-C. This causes an increase in neuronal excitability for tens of minutes even after the initial stimulus has stopped. This recruits responses to subthreshold stimuli and mediates the change from short-term to long term hyperexcitability of nociceptive spinal cord neurons. The increase in excitation along with a reduction in the inhibition of spinal cord neurons results in central sensitization^[10]. However, the neurons in the dorsal horn of the spinal cord are also excited by other second messenger pathways that are activated by brief and intense C-fiber signals^[53]. Within seconds of exposure to strong nociceptive stimuli, central sensitization develops, resulting in enhanced nociceptive facilitation that can persist for hours beyond the initial stimulus^[54].

There is evidence that stimulation of glial cells within the dorsal horn of the spinal cord also plays a crucial role in the creation and maintenance of pathological pain^[55]. When nerve trauma or inflammation occurs, glial cells become activated, which leads to the production of several proinflammatory mediators^[56,57]. Crucial pro-inflammatory cytokines include IL-6, IL-1b and TNF- α ^[56]. Chemokines such as monocyte chemoattractant protein and macrophage inflammatory protein-2 recruit neutrophils and macrophages from the bloodstream into the neurons^[57].

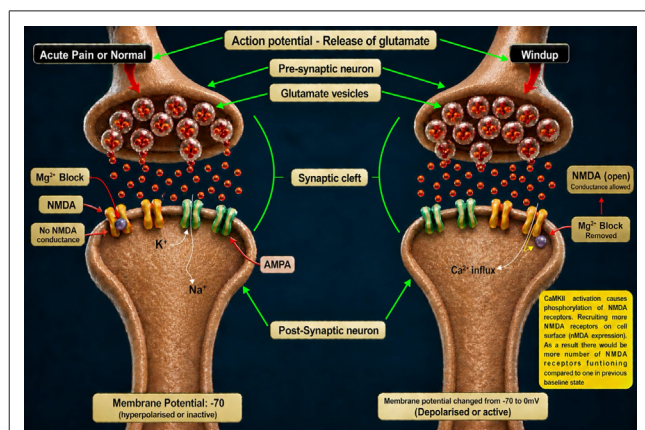


Fig 5. Illustrative summary of central sensitization compared with acute pain or normal nerve stimulus (Bombardment of WDR and nociceptive-specific neurons by afferent C fibers results in a progressive increase in the frequency of firing of second-order spinal neurons at a frequency above 0.5 Hz. This is also known as windup, in which magnesium blocking of the N-methyl-D-aspartate (NMDA) receptors is removed, rendering them for glutamate activation. Glutamate is the main and fast neurotransmitter in the CNS and plays a major role in nociceptive transmission. Glutamate acts at α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, NMDA receptors, kainate (KA) receptors, and metabotropic glutamate receptors. Glutamate-activated NMDA receptors are involved in the increased synaptic efficiency. Phosphorylation of NMDA receptor activates its mobility from intracellular store to synaptic membrane, enhancing its responsiveness to glutamate by removal of the voltage-dependent magnesium (Mg^{++}) ion block, thereby favoring the open-state configuration)

Thus, an increased pain state, central sensitization, can be established and sustained by these neuroexcitatory chemicals released after glial cell activation. Central sensitization results in allodynia and hyperalgesia by increasing the receptive field (response area) and tissue sensitivity to thermal, mechanical, and chemical stimuli^[58]. The difference between central and peripheral sensitization is that central sensitization allows low threshold sensory fibers (A β) to cause pain by increasing the excitability of spinal neurons and produces changes in spinal cord sensory processing. In contrast, peripheral sensitization enables low-intensity stimuli to induce pain by stimulating sensitized A δ and C fibers. In central sensitization pain signals are produced by low-threshold A β mechanoreceptors, which normally produce touch, vibration, and movement pressure (nonpainful) signals. Central sensitization has a crucial contribution to postsurgical hypersensitivity^[10].

Thus, surgical trauma contributes to postoperative pain hypersensitivity via peripheral and central sensitization: two different but collaborative mechanisms^[59]. In patients undergoing ovariohysterectomy, this phenomenon of peripheral and central sensitization has been demonstrated clinically by an increase in pain score in response to palpation at surgical and distant sites^[60-62].

LONG-TERM POTENTIATION

Long-term potentiation (LTP) has been reported as an important form of synaptic plasticity in nociceptive spinal pathways, and its mechanism resembles central sensitization. In acute and chronic pain states, it has been suggested as one of the cellular mechanisms contributing to pain amplification. A noxious stimulus is mandatory to develop a typical spinal LTP and therefore speculated to contribute to acute and chronic forms of pain that develop post-operatively or from an initial painful event, neuropathy or peripheral inflammation^[63,64].

LTP induction at spinal C-fibre synapses requires a rise in post-synaptic Ca⁺ concentration. Post-synaptic Ca⁺ rises by opening of the post-synaptic NMDA and Ca⁺-permeable AMPA receptors, T-type voltage-gated calcium channels and release of Ca⁺ from intracellular calcium stores. All these calcium sources trigger the activation of metabotropic glutamate or neurokinin-1 receptors. Finally, this rise in post-synaptic Ca⁺ activates Ca⁺-dependent signaling pathways involving calcium calmodulin-dependent protein kinase II (CaMKII), protein kinase C, and nitric oxide synthase (NOS)^[65-67].

Currently, the evidence is clear for the post-synaptic Ca⁺-dependent form of LTP induction in spinal cord lamina I neurons. Induction of LTP at C-fibre synapses requires activation of group I metabotropic glutamate receptors^[68]

and co-activation of the NK1 and NK2 receptors. It also requires the opening of ionotropic glutamate receptors of the NMDA type^[69-71] and T-type voltage-gated calcium channels^[70,71]. Substance P-mediated stimulation of NK1 receptors may directly augment single NMDA channel opening^[72] and NMDA receptor-mediated electric signals in lamina I neurons. All this may result into a significant increase in post-synaptic intracellular calcium^[70].

In an *in vitro* setting, it has been shown that an increase in post-synaptic intracellular Ca⁺ is crucial for LTP induction, and there is a linear correlation between the amount of Intracellular Ca⁺ increase and the amount of LTP^[61]. In a previous study, the data demonstrated that LTP-induced stimuli caused a significant rise in intracellular Ca⁺ in lamina I neurons, and this rise was not only observed during slice preparations but also in intact animals^[71]. Therefore, it was not surprising that signal transduction involves Ca⁺-dependent pathways, including activation of CaMKII, NOS, protein kinase A and C, phospholipase C, inositol triphosphate-3 (IP3) receptors and extracellular signal regulated kinase (ERK), including members of the mitogen-activated protein kinase family (MAPK)^[22,70,73-76].

In summary, significant similarities exist between central sensitization and spinal cord long-term potentiation, and both require NMDA receptors and involve key signaling transduction pathways, including extracellular signal-regulated kinase, protein kinase C, Src, and dependence of protein synthesis and gene transcription^[64]. The salient features of acute central sensitization are short latency (seconds) by intense, repeated, or sustained nociceptive stimuli, which typically can persist for several hours in the absence of further nociceptive stimulus^[77]. However, an additional mechanism may be needed for maintenance of late-phase long-term potentiation (greater than 4 h). Because it is technically challenging to follow long-term potentiation over time, it is unknown if there is ongoing spinal long-term potentiation, months after painful insults^[64].

NMDA receptors play a crucial role in the spinal pain process. Noxious and non-noxious stimuli stimulate the sensory dorsal horn neurons through NMDA. This suggests a role for NMDA receptors in nociception, as well as in the development of behavioral hyperalgesia and allodynia. Various NMDA antagonists reduced thermal hyperalgesia, mechanical hyperalgesia/allodynia, and spontaneous nociceptive behavior. Thus, surgical stimuli can trigger LTP in nociceptive pathways resulting in chronic post-surgical pain (neuropathic pain); clinically, manifested as hyperalgesia and allodynia^[78]. Understanding the NMDA, AMPA receptors, calcium dependent pathways, and other molecules involved in LTP, and drugs to antagonize these pathways could be a way forward to manage or prevent chronic pain.

Mechanism of LTP has been reported to be associated with crucial physiological functions such as learning, memory, cue reward-learning and fear in various regions in the brain, for example, in hippocampus, neocortex, thalamo-amygdala and lateral amygdala. Whereas, in the dorsal horn of the spinal cord LTP is associated with pathological pain or chronic pain^[79]. Lack of understanding of this association of LTP across various brain regions makes it challenging to design region-specific therapeutic interventions without affecting unrelated areas. Many molecular pathways involved in LTP might have a negative effect on other physiological processes, such as addiction potential, normal sensory processing, memory and learning. These could be possible challenges in translating LTP research into effective clinical therapies.

ASSESSMENT OF PAIN

Relieving the pain and suffering of the animals is the prime obligation of the veterinary clinician and researcher. It has been demonstrated that painful stimuli in humans cause comparable behavioral and physiological changes to those in other nonhuman species^[5]. Human verbal and physical communication help clinicians and researchers evaluate pain. The lack of this communication ability makes it difficult and challenging to assess pain. Dogs and cats likely perceive pain in a manner similar to humans due to the presence of comparable, if not highly conserved, neurotransmitters and neural pathways involved in nociceptive processing^[5]. Assessment and recognition of pain is an essential part of animal care and veterinary clinical practice. Quantitative assessment of pain in animals is a challenge to both researchers and clinicians and hinders objective measurement of the efficacy of analgesics. The ability to objectively measure pain is a pre-requisite to compare the efficacy of analgesics, which is difficult in animals because they cannot communicate directly^[80]. Various models/methods of pain assessment, including behavioural^[81], neurohumoral^[82] and neurophysiological^[83] have been used in veterinary research.

Basic Animal Pain Model

Research studies in humans and animals exploring the mechanism underlying acute pain as well as evaluating the analgesic effects of the drugs require appropriate use of stimulus to instigate the sensation. This stimulus must be non-invasive, repeatable and quantifiable^[80,84] to obtain an appropriate response. Electrical, mechanical, thermal and chemical stimuli are four basic types of nociceptive models used to study the analgesic effects of the drugs in question. The end point of these stimuli is the various withdrawal reflexes or behavioral responses^[80,84].

Electric Stimulation: A painful electrical stimulus can be helpful to assess the effectiveness of centrally acting

agents. Electrical stimuli are non-invasive, quantifiable and reproducible and produce synchronized afferent signals. However, there are some disadvantages of the electrical stimulus, such as it is not a natural stimulus felt by animals in a normal environment. Furthermore, it bypasses the transduction mechanism (circumventing the transduction process at sensory receptors) and directly stimulates all peripheral afferent fibers ($A\beta \rightarrow A\delta \rightarrow C$ fibers)^[85]. Electric stimulation has advantages in studies where effects of the drugs are assessed on the CNS, when administered systematically. Any effect will be of central origin because of the bypassing mechanism of the transduction process, provided that the drug has no effect on peripheral fibers. Variation in the impedance of the tissue being stimulated is another disadvantage; nevertheless, this can be minimized by using a constant current stimulator and monitoring the voltage and current of the stimulus applied. When the electrical stimulus is applied, it initially activates $A\beta$, followed by $A\delta$ and then C fibers^[80]. A double pain phenomenon has been reported in humans, the first or "fast", a well localized, stinging type of pain as a result of activation of $A\beta$ fibers and second or "slow", burning type of pain, more intense and difficult to localize, as a result of activation of C fibers^[86].

Various studies have reported the use of electric stimulation to evaluate a particular model and to evaluate the analgesic effects of test drugs. The electric current was used to stimulate the radial and tibial nerves, limbs, oral mucosa and periosteum to evaluate the microstrain gauge algometer for quantitative measurement of nociception^[87] and validate the noxious stimuli techniques for determining the minimum alveolar concentration (MAC) in dogs^[88]. Somatosympathetic reflexes of midazolam^[89] and clonidine^[90] were observed by electric stimulation of radial and tibial nerves in dogs. Electric stimulus was used on the dental pulp of the anaesthetized dogs to evaluate the analgesic effects of IV and intrathecal morphine compared to saline^[91]. To evaluate the analgesic efficacy of low-dose ketamine in conscious dogs, Bergadano et al.^[92] used a constant current device with a maximum voltage of 100 V and a maximal current of 40 mA to test a repetitive nociceptive stimulus. To evaluate the effects of tramadol, morphine and parecoxib on electroencephalography (EEG), Kongara et al.^[93] used an electric current of 50 V at 50 Hz for 2 seconds on the right hind limb of dogs. A similar stimulus has been reported by Kaka et al.^[94] in dogs.

In summary, various models have been developed for the quantitative evaluation of the antinociceptive effects of drugs in dogs. None of the models are ideal for use in dogs, with each having advantages and disadvantages. Nevertheless, these studies have demonstrated the use of electric stimuli in various models to assess the analgesic effects of drugs.

Mechanical Stimulation: In mechanical stimuli, pressure is applied against the surface of an organ to be tested. Application of the pressure initiates a nociceptive response; application of pressure can be progressive or coarse. The animal produces a behavioral response when the stimulus activates the nociceptors. These behavioral responses are the active withdrawal of the limb, guarding the test site or vocalization. The stimulus is withdrawn immediately at the time of an active response^[81].

Various models of mechanical nociception have been developed to evaluate the analgesic effects of drugs. Colorectal distension with a balloon was used in rats, a mechanical model of visceral nociception. Avoidance behavior along with reflex activities was recorded by electromyogram from the abdominal muscles; this model also developed tachycardia and increased arterial blood pressure. These parallel parameters were attenuated or even reversed using certain anaesthetic agents^[95]. Other similar models were distension of the stomach^[96] or duodenum^[97,98]. The model of colonic distension has also been reported in rabbits^[99,100] and dogs^[101].

Other different designs of mechanical devices have been used in dogs while working to quantify pressure applied to toes using a pair of Doyen intestinal forceps^[102], pneumatic device^[103,104] and aluminum pliers with concave working ends^[105]. The disadvantage of these techniques is that the rate of stimulus application cannot be controlled, and they can only be applied to the toes. In previous studies, an electronic method to measure joint tenderness in rheumatoid arthritis and tenderness of myofascial tissues was described by Atkins et al.^[106] and Bendtsen et al.^[107] respectively. Barnhart et al.^[108] used a pressure device for mechanical pressure in dogs. The device consisted of a pneumatic cylinder powered by compressed nitrogen. When pressurized a bolt (2.54 cm in length) protruded out of cylinder pressing the skin overlying the periosteum of the medial aspect of the proximal tibia with enough pressure to cause transient discomfort to the dog. This method could not demonstrate the antinociceptive effects of morphine.

Advanced Mechanical Models

In electronic measurement devices, the force applied via a rigid probe tip is quantified using an integrated load cell, which converts mechanical force into an electrical signal for precise measurement. A von Frey device works on the same principle and contains a tip that applies a gradually increasing force to the skin surface to create noxious stimuli. In some von Frey devices, fine hairs can also be used instead of a rigid tip that bent at a maximum force, and larger hairs can be used to retest until the subject reacts prior to hair bending. While working on the quantification of pain with a von Frey device on

humans^[80] scientists have diverted their attention towards developing devices that can be used at the site of pain. Recently, a von Frey device has been used successfully to measure mechanical nociceptive threshold testing for analgesic trials of morphine in dogs^[109]. For algometers refer to section- "Objective tools of pain assessment".

Thermal Stimulation: Heat causes selective stimulation of cutaneous receptors, resulting in specific activation of thermal and nociceptive fibers. This type of stimulation (with radiant lamps or contact thermodes and immersion of an animal's limb or tail in a thermostatic bath) results in the asynchronous stimulation of peripheral and central neurons because of the slow speed of cutaneous heating (<10°C/s). Thus, this model is not suitable for the study of a neural phenomenon seen in other sensory systems, where synchronous excitation of fibers is mandatory. However, thermal stimulation using a CO₂ laser thermal stimulator, results in the stimulation of nociceptive receptors only, as it does not actually contact the skin and provides controllable, safe, reproducible and specific noxious stimuli^[80].

Various models of heat sources have been developed and are being used in studies evaluating the analgesic efficacy of drugs. Barnhart et al.^[108] used the thermal model to assess the antinociceptive action of morphine. There was no significant difference in thresholds between the control and morphine groups. The device was comprised of a linear, ramped-intensity, incandescent bulb housed in a metal cylinder. The device had a slow rate of heating and was dependent on skin contact. These factors may have influenced the consistency and accuracy of the measured responses and should be considered when interpreting the study results. Recently, Hoffmann et al.^[110] used a thermal device containing a "thermal probe" as a temperature sensor and "heater element" in a thermally conductive epoxy that can be attached to the clipped thoracic wall of a dog or cat by an elasticated band in the center of an inflatable bladder that provides constant contact between the probe and the skin^[111]. The probe can be heated to record the skin temperature at a steady, incremental rate of 0.6°C per second until the dog responds to the thermal stimulation or the 55°C safety cut-out, whichever comes first. This model was used to assess the baseline reproducibility and the effect of levomethadone, acepromazine and fentanyl.

Chemical Stimulation: Chemical stimulation involves injection of an algogenic substance into the animal tissue. These tests use intraperitoneal and intradermal injections of irritant chemicals, such as formalin, capsaicin, carrageenan, turpentine and complete Freund's adjuvant (CFA), commonly available inflammatory substances with the ability to provoke inflammatory responses and irritating body tissues. This stimulation is very slow,

progressive, of longer duration and different from electric, mechanical and thermal stimuli. The result of this test is a behavioral score rather than the threshold measured. Animal models of visceral and peritoneal pain also use algogenic agents.

In a previous study, an administration of intradermal 0.5 - 15% formalin at 0.5 -15% on the dorsal surface of the fore paw of the rat produced painful behavior. This behavioral posture was graded as "0" for normal posture; "1" for the paw on the ground with no support to the animal; "2" for the paw unable to place on the ground; and "3" for the paw being licked, nibbled, or shaken in response to chemical irritation^[112].

The chemical nociceptive model uses the "writhing test" by injecting the algogenic substance intraperitoneally and directly into the hollow organs to produce visceral pain in rats^[113,114]. This pain is characterized by whole body movements (particularly of the hind paws), abdominal contractions, twisting of the dorsoabdominal muscles, and decreased motor activity and motor incoordination. The "pain behaviour" produced by these tests is complex and biphasic, characterized by body stretching and contraction of either the flanks or the whole body in the initial phase and predominantly involves abdominal licking and nibbling in the second phase^[115,116]. Similar models have been reported for bladder pain with intravesical injection of capsaicin, capsaicin-like substances^[103,117-119] and turpentine as an irritant substance^[120]. A model of uterine pain was also developed, where intrauterine injection of mustard oil was used as an algogenic agent^[121].

In an experimental model, the dogs under anaesthesia were given sodium urate or calcium pyrophosphate crystals in stifle joints to quantify the host response^[122]. Toutain et al.^[123] used Freund's adjuvant arthritis model of pain in dogs to determine the dose regimen of nimesulide, a COX-2 selective nonsteroidal anti-inflammatory drug (NSAID). In another study^[124], efficacy of low-dose ketoprofen was investigated in sodium urate crystal-induced synovitis model of arthritis. In a similar study by McCann et al.^[125] a urate crystal-induced synovitis model was used to observe the *in vitro* effects and *in vivo* efficacy of a novel COX-2 inhibitor in dogs.

In summary, the above studies demonstrate the use of animal models of pain using various chemical agents in rats and dogs. The disadvantage of these models is that they may harm the animal because they have unavoidable characteristics once administered^[80]. Additionally, chemical models are more invasive and less humane^[80]. These models are experimental models of chronic pain. Therefore, distinction should be made from our current discussion on acute perioperative models. Detail discussion of the chronic models of pain is beyond

perioperative pain. It is worth to mention here that, the fundamental knowledge on pain studies was developed from rodent models, however, the disparity between nerve fibers composition, density of nerve fibers per skin area, and pain tolerance may lead to key differences in pain response among dogs and cats from rodents^[126].

Surgical Models of Pain

Ovariohysterectomy and Castration: Over the last decade, interest in the understanding of the mechanism underlying pain for the benefit of animals has increased. For this purpose, most of the work has focused on acute pain models. Commonly used models for this were heat or pressure^[127,128] and clinical pain^[129]. Most acute clinical pain studies have evaluated the effects of surgical trauma on companion animals^[93,94,128,130]. Among these studies, the most commonly used surgical model of pain is ovariohysterectomy. The Center for Veterinary Medicine of the US Food and Drug Administration (FDA) considered OHE a suitable model to investigate moderate pain in clinical studies of analgesia^[129]. Castration and ovariohysterectomy are commonly performed elective surgical procedures in small animal practice^[131]. These surgeries produce significant postoperative pain^[132]. Several studies have reported ovariohysterectomy, to be a noxious procedure for bitches^[129,133,134]. Traction on the mesovarium during ovariectomy is recognized as one of the most potent noxious stimuli, often eliciting pronounced autonomic and hemodynamic responses, and, in some cases, patient movement despite anesthetic depth^[135,136]. The efficacy of the drug to be tested can be evaluated more reliably on these elective procedures because these surgeries are performed on healthy animals that are pain free, assuming all the postoperative pain is due to surgery^[137].

PAIN ASSESSMENT TOOLS

Pain assessment tools can be broadly categorized based on their application in experimental or clinical settings and may be further divided into objective and subjective measures.

Objective tools include neuro-endocrine responses, heart rate (HR), blood pressure (BP), respiratory rate (RR), electroencephalography, heat and mechanical threshold testing and hand-held devices such as algometer and the von Frey device. Heart Rate, BP, RR and pupillary diameter are routinely used parameters of pain assessment intraoperatively as well as postoperatively during the recovery period and even later. Postoperatively, factors such as stress, fear, and environmental noise may also

influence physiological parameters, including HR, BP, and RR, and should be considered and controlled for when interpreting these measurements [138-140].

Objective tools include behaviour and pain scoring scales. Objective markers like HR, BP and RR would be very useful along with behavioural markers for proper pain assessment. Pain scoring scales can be further categorised into unidimensional and multidimensional. Unidimensional scale consists of expressions like No, Mild, Moderate or Severe Pain. Examples of these include simple descriptive (SDS), Numerical Rating Scale (NRS) and Visual analogue scale (VAS). Multidimensional are subcategorised with behavioural or pictorial indicators given a particular score, these include the University of Melbourne Pain Scale, Glasgow pain scale, Colorado pain scale for canine and feline, Feline Grimace and UNESP-Botucatu MCPS. The advantage of multidimensional scales is that they allow pain measurement from more than one aspect; neither specialized skills nor experience is required to use these scales. This could be due to the pain descriptors, acquired from practicing veterinary surgeons, regularly examining dogs and cats for acute pain behavior.

Requirement of busy veterinary practice is that a pain scale should be user friendly, require less skills and knowledge for scoring. The authors' experience with the Glasgow Composite Pain Scale and, more recently, the Feline Grimace Scale has been favorable. In contrast, the Visual Analog Scale was found to be more challenging to apply consistently.

OBJECTIVE TOOLS OF PAIN ASSESSMENT

Neurophysiological Techniques

Electroencephalography: Electroencephalography (EEG) has been used as an objective tool for the investigation of pain [141]. This is a non-invasive and stress-free technique. Parameters of the EEG for nociception under anaesthesia and pain in conscious animals have received much attention. However, due to the deficiency of standard recording techniques in animals, and complexity in data analysis, the use of EEG in veterinary practice is limited [142].

What is Electroencephalography? The EEG is the real-time graphical representation of the microvolt range tiny but spontaneously produced neuronal signals from the cerebral cortex through electrodes placed on different positions on the head. Neurons having an intrinsic electrical nature to produce electrical and magnetic fields. When recorded at a distance from the source, these fields are termed "far-field potentials", whereas those recorded at a short distance from the source are known as "near-field

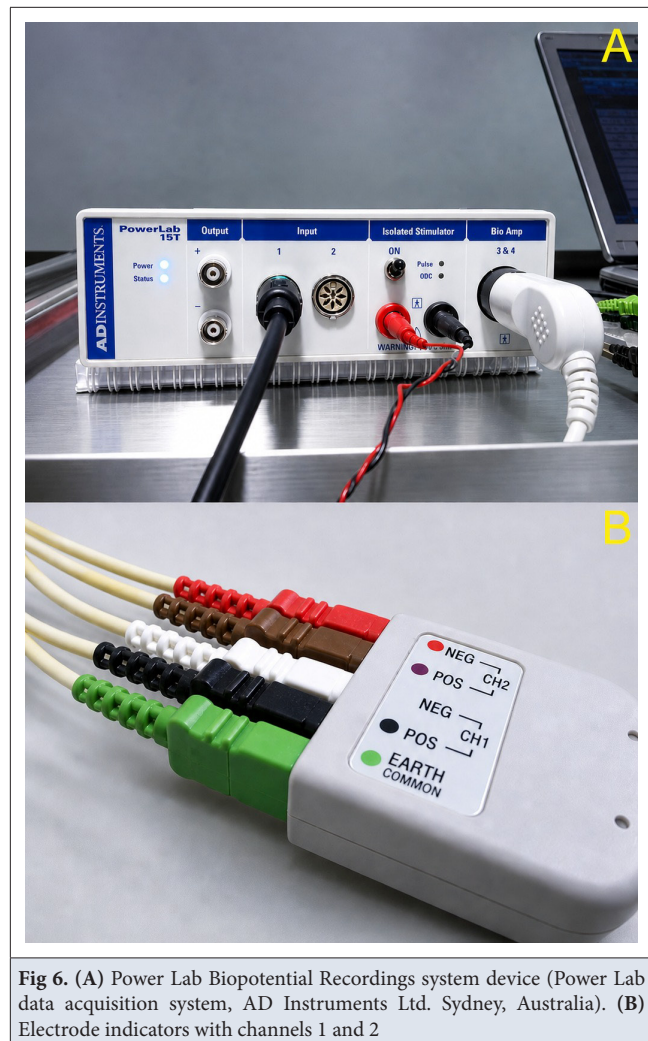


Fig 6. (A) Power Lab Biopotential Recordings system device (Power Lab data acquisition system, AD Instruments Ltd. Sydney, Australia). **(B)** Electrode indicators with channels 1 and 2

potentials". The EEG is recorded at distance from the source neurons and therefore is "far-field potential". An action potential transmitted along the nerve fiber approaches a synapse and creates a change in the membrane potential of the postsynaptic neuron, the postsynaptic potential. These changes in the membrane potential of postsynaptic neurons are the main contributors of the EEG [143].

Electroencephalography Recording: The EEG recording apparatus comprises electrodes, a signal recording amplifier and a recording system (Fig. 6). Electrodes connect with the conducting fluid of the tissue and the input circuit of the amplifier. The impedance is crucial for accurate and/or minimum loss of EEG signals. Thus, electrode-tissue impedance is maintained at a level substantially lower than the input impedance of the amplifier to ensure accurate signal acquisition [141,143,144]. Different configurations for the placement of the electrodes have been described, and several studies have reported the configuration described by Mayhew and Washbourne [145] with the positive, negative and ground electrodes as shown in Fig. 7 [142].

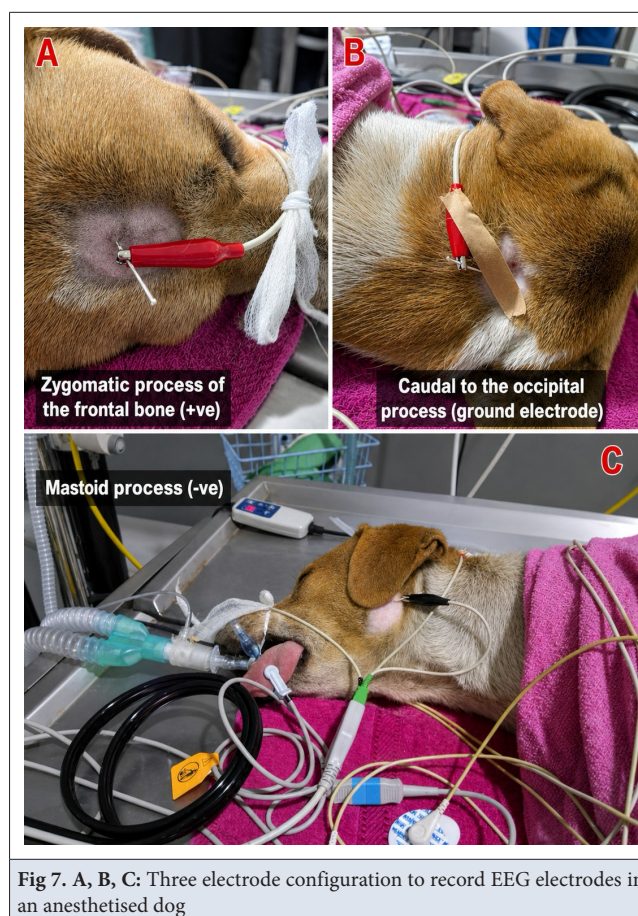


Fig 7. A, B, C: Three electrode configuration to record EEG electrodes in an anesthetised dog

Analysis of the Electroencephalographic Data: The analysis of EEG data is conducted offline at the end of the experiment. Fast Fourier Transformation (FFT) is a mathematical procedure to quantify data within raw EEG signals. The fast Fourier transformation system converts the raw EEG signals to different frequencies characterized by corresponding amplitudes. These EEG frequencies have been reported as delta (0.1 to 4 Hz), theta (4.1 to 8 Hz), alpha (8.1 to 12 Hz) and beta frequency [12.1 to 20 Hz] [142,143,146]. This creates a power spectrum, which is the distribution and derivation of the EEG variables of corresponding frequencies and amplitudes (Fig. 8).

Variables of the Electroencephalography: Total power (Ptot), Median frequency (F50 or MF) and spectral edge frequencies (SEF95%) are the variable extracted from EEG data. These are the most common variables associated with nociception in animals [142,144,146]. "Total area under the cover" is the total power. The median frequency is "the frequency below which 50% of the total power of the EEG is located". Spectral Edge frequency 95% is "the frequency below which the 95% of the total power of the EEG is located" [144].

Arousal or desynchronization is characteristic of the brain's response to unpleasant stimulus, typically causing increase in MF (Fig. 9) and a decrease in Ptot [93,94].

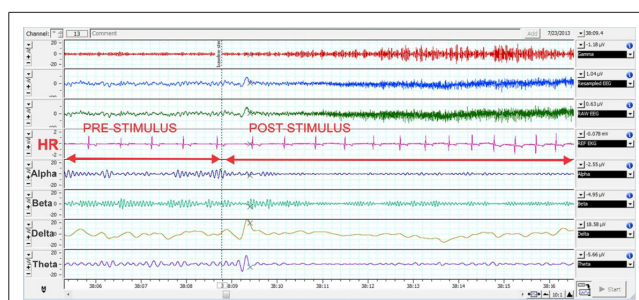


Fig 8. EEG spectrum of a dog under anesthesia, showing visible effects of nociceptive stimulus (pre versus post stimulus) on various waves and heart rate

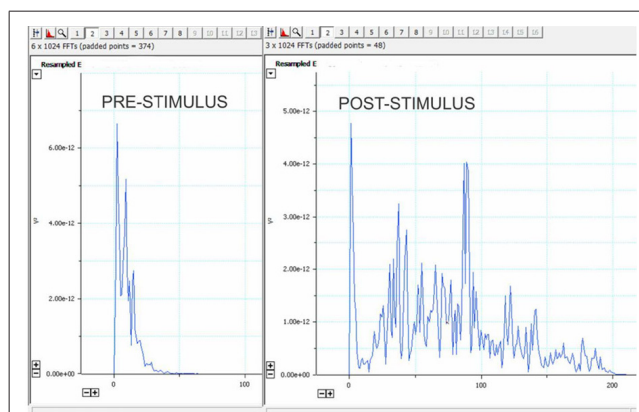


Fig 9. Median frequency of a dog under anesthesia, showing visible effects of nociceptive stimulus before and after stimulus. Note the increase in the area under the curve after stimulus

Noxious stimulus results in a change of the EEG from high amplitude low frequency (seen in anaesthesia, sedation, sleeping or calmness) to low amplitude high frequency waves (resembling that of awakesness or alertness); this shifting of EEG is called arousal or "desynchronization" [144].

Synchronization or paradoxical arousal is another change in EEG associated with noxiousness but is less commonly reported and is mostly described in clinical studies, which used multiple anesthetic and analgesic agents with varying noxious stimuli [147-149]. Synchronization is a shift in the EEG towards high-amplitude and low-frequency activity. Some other clinical studies were unable to demonstrate EEG changes in response to noxious stimuli [150-152]. However, current reports suggest that EEG is a robust tool for well-controlled nociceptive studies [141,142,143,146].

Changes in EEG Spectrum During Nociceptive Studies:

It has been accepted that EEG is a direct indicator of the state of responsiveness of the central nervous system under anesthesia [94,142]. Changes in the EEG power spectrum can be used objectively to quantify nociception in horses [153,154], rats [155], deer and sheep [156,157], calves [158] and dogs [141,142]. Median frequency has been reported to be the predominant change in response to noxious

stimuli [93,94,141,142]. These lines of evidence suggest that EEG can be used as an objective tool to evaluate the analgesic efficacy of drugs.

Minimal Anesthesia Model (MAM)

Johnson and his co-workers developed a model of anesthesia to study the effects of noxious stimuli on the EEG spectrum [144]. This model was first used in horses to investigate the effects of castration on EEG [153]. In the minimal anesthesia model, animals are anesthetized to a stable plane, but they are capable of showing EEG responses to nociceptive stimulation despite being unconscious. When compared with other studies using painful methods, this model works a negative control group without compromising animal welfare/ethics. This method uses the EEG response to noxious stimulation as a technique to assess analgesia for studies of pain in applied veterinary research. This is a sensitive and quantitative technique of assessing central responses to painful stimulation [144]. A minimal anesthesia model has been used in horses [153,154], sheep [156], red deer [157], pigs [160] and dogs [93,94,141,142].

Somatosensory Evoked Potentials

Somatosensory evoked potentials (SEPs) have been reported to evaluate the function of the nervous system. These are the electric potentials generated at various levels of the nervous system, for example peripheral nerve, plexus, nerve roots, spinal cord segments and the sensory cortex. Most commonly used nerves for the stimulation of pelvic and thoracic limbs are tibial and median nerve, respectively [161-164]. These nerves are stimulated through inserting electrodes or stainless-steel needles into subcutaneous tissue at distal end. Stimulation at proximal to targeted nerve can result in more reflex actions of muscles due to recruitment of more fibers with increased potential. This increased activity of the muscles can be avoided by stimulation of the median nerve just above the carpus and tibial nerve just above the tarsus [151]. The cathode (negative electrode) can cause depolarization of the axon membrane and should be placed 2 cm proximal to the anode (positive electrode), to avoid conduction block by anode [162,163]. Recordings of the evoked potential are shown on a computer screen; recordings and waves associated to each limb can be seen in various windows, adjustable for improved visualization. Clinically SEPs are used to evaluate the integrity of sensory pathway conductivity and may therefore be used to examine central and peripheral neurological conditions [163].

von Frey

In electronic devices, an integrated load cell is used to measure the force applied to a rigid tip. A von Frey device consists of a tip that produces painful stimuli by



Fig 10. Wagner algometer FPX 25 with circular tip used in various studies of pain research in animals

gradual application of force to the skin surface. Some von Frey devices employ larger hairs to retest until the subject reacts before the hair bends, whereas fine hairs are used that bend at a maximum force. While working on quantification of pain with von Frey device on humans [80] scientists have diverted their attention towards developing devices which can be used at the site of pain. von Frey has been used successfully to measure mechanical nociceptive threshold testing for analgesic trials in dogs [110].

Algometry

Schiffman et al. [165] described a pressure algometer for examining muscle tenderness in patients with myofascial pain syndrome. The use of algometers for mechanical pressure in humans led to the usage of algometers for mechanical nociceptive threshold testing of analgesic trials in dogs [61,129,166]. An algometer consists of a probe with a tip or disc, a load cell, transducer and an amplifier. It is used perpendicular to the skin on the point to be tested. Pressure is applied on the site by gradually increasing force to produce a painful stimulus (Fig. 10). The force exerted to the tip is transferred to a load cell, producing a voltage output. This voltage output is transduced, amplified, and

displayed in newtons or kilograms^[61,128,166,167]. These devices have the ability to activate both the A δ and C fibers, responsible for encoding clinical pain^[80]. Algometry can be used in clinical settings to assess post-operative hyperalgesia and in experiments to assess the effectiveness of analgesics^[168].

A variety of algometers have been used in humans^[169,170] as well as animals^[167,171-173] with different objectives. These studies have used various diameters of tips, such as 1 cm probe tip^[67,174], 2 mm probe tip^[61,132,171], and 0.5 mm probe tip^[109]. Due to various tip sizes, the pressure required to initiate the response was different in these studies. For example, pressures were 1.92 N^[109], 30.81 N^[171], ~38 N^[174,175], 38-44 N^[167], and 9.94 N at the tibia^[61] to provoke a response in these studies.

Regardless of the tip size, whenever an algometer is used in research, one should practice its use in order to avoid incorrect readings and then set a baseline of the particular experimental animal. Each animal would have a different baseline based on animal's pain tolerance. Before we begin to use the algometer in a clinical setting, there is a need to fix an end-point for a particular species. However, the use of an algometer is rare in clinical settings, due to the fact that behavioural and physiological parameters like HR, BP and RR can easily be applied for pain assessment. On the other hand, algometer is more frequently used in experimental models.

Fixing an end-point of a nociceptive test would be challenging experimentally because of individual or species variation. Peripheral perception may be impacted by nociceptors' distribution, blood flow, and skin thickness^[126]. Therefore, to achieve the best outcomes, it is essential to assess nociceptive threshold and determine the end-point for each species and individual. These end-points could be identified through training of the animals before the experiment. Vocalization, limb withdrawal reflex with and without vocalization and guarding of the abdomen were the most common end-points in dogs^[166].

Electronic von Frey and algometer have similar working principles. Unlike the von Frey device, the algometer is easy to use and consists of a single, basic handheld unit without any electrical wire or additional parallel units. It can be used easily in the clinical setting in conscious dogs. One of the disadvantages of the algometer use is the inability of the operator to apply a continuous rate of force. Threshold readings could be affected by the rate of force application, for instance, faster rates would result in higher readings^[176]. This can be minimized by continuous practice^[62] or using a continuous rate of application of force (CRAF) meter for accuracy^[61].

Neuro-Endocrine Responses

Endocrine response is stimulated by afferent action

potential from the site of surgery. These action potentials travel along sensory nerve roots through the dorsal root of the spinal cord to the medulla to stimulate the hypothalamus. Increases in sympathetic activity and pituitary hormones are characteristic responses to surgery^[177]. Norepinephrine from the presynaptic nerve terminals and catecholamines from the adrenal medulla are released as a result of hypothalamic stimulation of the sympathetic autonomic nervous system. Hypothalamic releasing factor stimulates the anterior pituitary, causing the secretion of adrenocorticotrophic hormone (ACTH), growth hormone, prolactin, arginine vasopressin and beta-endorphin^[178]. Growth hormone activates protein synthesis and prevents its breakdown, activates glycogenolysis in the liver, promotes lipolysis, and has an anti-insulin effect^[177]. Arginine vasopressin, which has a major role as an antidiuretic hormone, is also released from the posterior pituitary. Corticotrophin or adrenocorticotrophic hormone acts on the adrenal cortex and increases cortisol secretion in the plasma within minutes after surgery. Both of these hormones can be measured in the plasma within minutes of surgery^[177].

Cytokines: Surgery instigates a series of events, including inflammatory reactions and nociception. Tissue and peripheral nerve injury can result in a local inflammatory reaction. In reaction, the concentrations of various biological mediators, including substance-P, PGs, bradykinins, CGRP and cytokines, can also be elevated in the injured tissue^[179], particularly pro-inflammatory cytokines^[180,181]. Among various mechanisms of pain, pro-inflammatory cytokines have recently been investigated comprehensively^[180,182,183].

Cytokines are small proteins secreted and released by cells. They have specific action on the interaction and communication between cells. Cytokines are produced by cells such as lymphocytes (lymphokines) and monocytes (monokines). Those produced by leukocytes act on other leukocytes (interleukin), and cytokines can also release chemokines to perform chemotactic activities. Cytokines are pleiotropic (various cell types produce the same cytokine or single cytokine acts on different cell types) as well as redundant (same functions stimulated by different cytokines) in their action. They are mostly released in a cascade where one cytokine activates its target cell to produce additional cytokines. They also have synergistic and antagonistic effects. Many cells are responsible for making cytokines, but macrophages and helper T cells are principal cells^[184].

Mechanism of Action: Cytokines influence the intracellular modulating processes by binding its specific membrane-bound receptor. A series of phosphorylated constitutively expressed signal proteins takes place inside the cell. These phosphorylated signal proteins travel through

the cytoplasm and contact the nucleus on the condition that they should have a nuclear localization sequence or bind to a protein with their compatible sequence. In the nucleus, they may affect the transcription rate or induce post-translational changes. Proteins that participate in pain are signal transducer and activator of transcription (STAT) [185], c-fos [186,187], Ras/Raf, c-Jun [186] and mitogen activated protein kinase (MAPK) [185,188]. Interestingly, these proteins also take part in the intracellular signaling pathways of several cytokines, including IL-6 [189].

In previous studies of the cellular mechanism of inflammatory pain, it has been established that a well-defined sequential cascade of cytokines/chemokines is followed by the release of PGs and sympathetic amines [190,191]. Classical pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 play roles in pathological pain [184]. IL-1 β and IL-6 can provoke peripheral and central sensitization, resulting in pain augmentation (hyperalgesia) [192,193]. The direct action of cytokines on nociceptive neuronal receptors and ion channels has been described in previous studies, where they regulate neuronal excitability, sensitivity to external stimuli and synaptic plasticity [194].

Exogenous Cytokine Response: In various experiments conducted on animal models, exogenous administration of pro-inflammatory cytokines augmented nociception [190,195-199] and increased neuronal excitability in response to noxious stimuli. In contrast, when the function of these cytokines was blocked with soluble TNF receptors, interleukin-1 receptor antagonist (IL-1ra), anti-IL-1 or anti-IL-6-neutralizing antibodies, it prevented or reversed hyperalgesia and allodynia in an animal model tested for peripheral inflammation and/or injury [182,200-203]. Patients with decreased levels of pro-inflammatory cytokines have reported less severe pain [183,204]. IL-1 and IL-6 are released in both the brain and periphery after exposure to psychological stressors [205].

Cytokine Response to Analgesic: Elevated central and peripheral levels of COX-2 and pro-inflammatory cytokines, especially IL-1 β , result in the production of prostaglandin E₂ (PGE₂) [206,207]. Pre-emptive epidural analgesia has been proven to reduce postoperative pain and decrease the production of pro-inflammatory cytokines, including IL-1 β [183]. Pre-emptive blockade of pro-inflammatory cytokines (TNF- α and IL-6) increased the nociceptive threshold in experimentally induced inflammatory pain [208], reduced morphine requirement and recovery was better after cancer surgery [209]. Decreased levels of proinflammatory cytokines have been observed in patients administered with mixture of opiates and local anesthetics in the postoperative period [45].

Role of Cytokines in Neuropathic and Inflammatory Pain: Neuropathic pain results from injury or dysfunction of

nerves. Animal models of neuropathic pain has shown involvement of several proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-17. These cytokines have been reported in disc herniation, sciatica, lumbar herniation, spinal cord injury and complex regional pain syndrome (CRPS) [210].

Possible mechanisms for IL-1 β , include activation of the dorsal root ganglion neurons and increased spinal cord activity. IL-7 results an increase in the activity of transient receptor protein vanilloid 4 (TRPV4), an ion channel that has been found to mediate mechanical allodynia.

Inflammation is caused by abnormal signaling or production of pro-inflammatory cytokines such as TNF- α and IL-6, IL-1 [211]. These cytokines are released by immune cells after tissue injury and inflammation, sensitizing peripheral nociceptors and reducing their threshold of stimulation. This stimulation triggers the action potential travelling to dorsal horn of the spinal cord, synapsing with second order neuron. Glutamate and substance P transmit pain signals to ascending pathway, reaching higher centers of brain involved in perception. At the same time modulation of pain occurs through descending pathways, affecting intensity and perception of pain [212]. Increased sensitivity of nociceptors and exacerbated pain responses are hallmarks of inflammatory pain, indicating involvement of both peripheral and central components of pain. IL-1, and TNF- α play a role in hyperalgesia. Development of therapeutic agents, targeting these cytokines and their signaling pathways would significantly help alleviate pathological pain. This offers a way forward as a therapeutic approach and requires understanding interplay between cytokines and various inflammatory or neuropathic conditions

Anti-inflammatory Cytokines: Just as proinflammatory cytokines and their involvement in pain, nature has provided anti-inflammatory cytokines to keep balance in the immune system by suppressing inflammation and maintaining immune homeostasis. when proinflammatory cytokine levels rise in neuropathic and inflammatory pain, there is decrease in the anti-inflammatory cytokines. Lower levels of anti-inflammatory cytokines have been demonstrated in patients with chronic neuropathic pain conditions, such as complex regional pain syndrome, atypical facial pain, lower back pain, and post-herpetic neuralgia. IL-4, IL-10, IL-13, transforming growth factor beta (TGF- β) [210], IL-35, and IL-37 [213,214] have been reported as anti-inflammatory cytokines. interleukin-10 and transforming growth factor-beta (TGF- β) limit the transmission of pain.

Glatiramer acetate, an immune modulator, has been reported to decrease pain and has been correlated with increase IL-4 and IL-10. Calcineurin, uliastatin,

plasmid DNA, and viral vector indirectly increase IL-10 concentrations and could be therapeutic target for neuropathic pain. Flexibilide, a substance derived from soft coral and delivery of bone marrow stromal cell into the spinal cord of mice has been reported to reduce neuropathic pain. Ibudilast, a phosphodiesterase inhibitor originally developed as an asthma medication, suppressed glial cell activation and reduced IL-1 β , TNF- α , IL-6 [210]. Botulinum toxin (BoNT), the paralytic neurotoxin produced by the bacterium *Clostridium botulinum*, reduced pain related to spinal cord injury [215] and peripheral neuropathic pain [216].

IL-35 is an anti-inflammatory cytokine with potent immunosuppressive capacity. IL-35 alleviated pain in autoimmune encephalomyelitis (EAE) mouse model of central neuropathic pain [217] and the streptozotocin rat model of diabetic neuropathy [218]. IL-37, suppresses inflammation and immune activation. Inhibits production of IL-1 β , IL-6, TNF- α . This anti-inflammatory cytokine has potential therapeutic target for autoimmune diseases and chronic inflammatory conditions [219].

Currently FDA approved TNF- α inhibitors include infliximab, etanercept, adalimumab, certolizumab pegol, and golimumab. These are recommended for painful disorders such as inflammatory bowel disease, rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis. Canakinumab, Gevokizumab, and Anakinra, are FDA approved drug against IL-1 β , Tocilizumab, Sarilumab, and Sirukumab against IL-6 (Low back and leg pain) and secukinumab for IL-7 and Secukinumab for IL-17. Other cytokine modulators include Emapalumab, Ruxolitinib, Tofacitinib, approved by FDA for various conditions involving pathological pain pathways [211].

Hormones (Adrenaline, Nor-adrenaline, Endorphins, Cortisol): Briefly, several hormonal changes linked with stimulation of the hypothalamic-pituitary-adrenal axis in response to surgical stress have been reported. Of these, adrenal corticosteroids such as cortisol, inflammatory mediators including beta-endorphins and other associated pituitary hormones have been used in previous studies of pain and associated stress assessment [82,128,130,220,221]. This method of changes in plasma concentration of hormones and cytokines is advantageous in the sense that it minimizes observer variability associated with behavioral assessment of pain and is more objective than behavioral assessment. However, there are some disadvantages and limitations [222] of this method, summarised as follows:

1. A single sample collected at a specific time point in an animal experiencing pain may not accurately reflect the level of pain present at that moment. For example, similar biomarker readings observed in control animals without pain and in animals subjected to painful stimuli may

reflect the inherent variability of hormonal and cytokine responses. These variations can result from changes in plasma concentrations associated with stress or other non-nociceptive physiological processes.

2. These biomarkers lack sufficient specificity for accurate pain assessment and should therefore be interpreted in conjunction with other validated scoring systems. However, their routine use in clinical practice is limited due to their invasive nature, associated costs, and the need for specialized analysis, and they are consequently infrequently utilized in clinical settings.

CONCLUSION

It is now well established that surgical trauma results in nociception triggering a cascade of chemical reactions that take place at the primary site and throughout the central nervous system. These chemical reactions produce an “inflammatory soup” that brings changes in the central nervous system, resulting in the development of central sensitization and long-term potentiation manifested clinically as hyperalgesia and allodynia, subsequently developing into chronic pain. Nociception includes sodium and potassium channels that contribute to transduction and transmission. Excitatory neurotransmitters, including glutamate, substance P and CGRP, play key roles in modulation. Glutamate, through activation of NMDA receptors and other excitatory pathways, plays a critical role in the development of central sensitization. The use of multimodal analgesia, involving drugs with different mechanisms of action, can help attenuate these processes and improve postoperative pain management.

DECLARATIONS

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

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RESEARCH ARTICLE

Targeted Prebiotics in the Control of *Eimeria* spp. to Reduce Concurrent Parasitic Zoonotic Animal Diseases in Poultry

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Abstract

Avian coccidiosis, caused by protozoan parasites of the genus *Eimeria* remains one of the most economically devastating diseases in poultry production worldwide. Traditional methods of control are mostly based on the use of anticoccidial medication and vaccines; nevertheless, the growing development of drug-resistant strains of the parasites and governmental limitations on the usage of chemotherapeutics have initiated the development of alternative methods of control. Because of the rising concern of antimicrobial resistance, there is a need to develop alternative control strategies. There is an emergence of targeted prebiotics as promising functional feed additives that can be used to regulate the microbiota in the intestine, improve the strength of the mucosal immune system, and improve the integrity of the gut barrier. Prebiotics help to increase intestinal homeostasis and coccidial protection through selective stimulation of positive microbial populations. Moreover, because of better gut health, there could be reduced vulnerability to concomitant parasitic zoonotic pathogens, including *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Giardia duodenalis*. This review investigates the application of targeted prebiotics such as fructo-oligosaccharides, mannan-oligosaccharides, inulin and β -glucans in the reduction of *Eimeria* infections, poultry performance, and in sustainable poultry production and poultry food safety.

Keywords: *Eimeria*, prebiotics, poultry nutrition, coccidiosis, control, gut microbiota, zoonotic parasites

INTRODUCTION

The world poultry business has become very important in supplying the increasing demands of animal protein ^[1]. Poultry meat and eggs are a major source of inexpensive human foodstuffs for a developing human population ^[2]. Nonetheless, infectious diseases remain a major impediment to the health, production, and financial viability of poultry ^[3]. One of the most common and costly diseases affecting poultry in the world is avian coccidiosis, which is caused by protozoan parasites of genus *Eimeria* ^[4]. Coccidiosis is an intestinal infection caused by ingesting sporulated oocysts in litter, feed, or water contamination ^[5]. After being consumed, the parasite has several developmental stages which occur inside the epithelial cells that form the intestinal tract ^[6]. Some species of poultry, such as *Eimeria tenella*, *Eimeria maxima* and *Eimeria acervulina*, all of which attack particular parts of the intestine ^[7]. The consequences of infection include the destruction of epithelial cells, haemorrhage, malabsorption, a decrease in growth performance, and an enhanced susceptibility to secondary

infection ^[8]. Coccidiosis may result in severe mortality cases, especially in young birds in severe cases ^[9].

The economic cost of avian coccidiosis is high and includes dwindling productivity, an upsurge in the ratios, death rates, and the expenditure on control ^[10]. The poultry industry has over the decades been majorly depending on anticoccidial drugs and vaccines to control the disease ^[11]. The chemical anticoccidials and ionophores have been extensively used in poultry feed to deter or restrict the reproductive ability of parasites ^[12]. Nevertheless, persistent and extensive use of such compounds has led to the development of *Eimeria* strains resistant to drugs and drug residue problems ^[13]. Moreover, the demand of consumers towards the antibiotic-free poultry products and the development of more stringent regulatory measures in most countries have restricted the use of chemotherapeutic agents in animal production systems ^[14,15]. The challenges have prompted the need to seek alternative methods for the control of coccidiosis that are safe and sustainable ^[16]. The alternative control strategies include use of botanical compounds, nanoparticles, probiotics, prebiotics, peptides, immunoglobulins, vaccines, vitamins



and many more that can be used in avian coccidiosis and multiple other parasitic, bacterial, and viral diseases [17-19]. The effectiveness of a functional feed additive to enhance the condition of the gut and host resistance against pathogenic organisms is one such promising strategy [20,21]. The prebiotics are one of them, which have acquired significant coverage in the scientific community as they possess the capacity to selectively activate health-promoting microorganisms in the gut and enhance the function of the intestine [22].

Prebiotics can be described as non-digestible dietary factors that have health advantages to the host through their selective stimulatory effects on desirable microorganisms in the gastrointestinal tract [23]. Fructo-oligosaccharides, mannan-oligosaccharides, inulin and β -glucans are most commonly used as prebiotics in poultry [24]. These are substances that are not degraded by host enzymes but fermented by beneficial microbes along the intestine, leading to the generation of short-chain fatty acids and other metabolites, which increase the health of the gut [25]. In the intestinal microbiota, the main role is to ensure the efficiency of digestion, immune functions, and defence against infectious agents [8]. A healthy microbial environment can prevent the settlement of pathogens by competitive exclusion, generation of antimicrobial compounds, and activation of host defences [26]. On the other hand, intestinal dysbiosis may make birds susceptible to illness and undermine overall productivity [27]. *Eimeria* infection impairs intestinal integrity and changes the composition of the gut microbiota, establishing advantageous conditions in the intestine for opportunistic agents and secondary infections [28]. A promising approach to alleviate the effects of coccidiosis is the enhancement of the intestinal microbial balance by dietary interventions (e.g., specific prebiotics) [29]. Prebiotics can improve the mucosal immune system, improve the efficiency of the epithelial barrier, and slow the growth of parasites by selectively boosting the beneficial microbial populations, including *Lactobacillus* and *Bifidobacterium* [30]. Besides controlling *Eimeria* infection, there is also the possibility that better gut health reduces the chances of concomitant parasitic zoonotic infections in poultry [31].

The poultry production systems are related to various parasites of zoonotic significance: in particular, *Cryptosporidium parvum* has been identified as a major foodborne zoonotic disease [32]. They may contaminate the environment and products of poultry and threaten human well-being [33]. Dietary interventions in enhancing the intestinal defence system of poultry could lower the shedding of pathogens and contamination of the environment, and thus, result in better food safety [34]. The most recent studies have shown that specific prebiotics can tune the host immunity, alleviate gut microflora

balance, and enhance resistance to illness in poultry [35]. In addition, the compounds can be used in combination with other alternative strategies, including probiotics, phyto-genic additives, and vaccines to increase the overall disease control [36,37]. The recent development of interest in microbiome-based strategies is indicative of a wider adoption of sustainable and antibiotic-free systems of poultry production [38]. Although there is a growing amount of evidence supporting the effectiveness of prebiotics, but their mechanisms need to be evaluated for effective use [39]. More studies are needed to clarify the interaction of dietary ingredients and intestinal microbiota, host immune response, and the development of parasites [40]. The objective of this review is to provide a comprehensive overview of the role of targeted prebiotics in controlling *Eimeria* infections in poultry.

GUT HEALTH AS A CENTRAL COMPONENT OF DISEASE RESISTANCE

Not only is the gastrointestinal tract of poultry the major place where digestive processes and the absorption occur, it is also an essential barrier against pathogenic microorganisms [41]. Healthy intestinal environment is the primary basis for keeping the overall health of poultry health, improving its growth performance, and preventing infectious diseases [8,42]. Gut health can be defined as structural integrity of intestinal epithelium, intestinal microbiota, balance and mucosal immune system efficiency [43]. The intestinal epithelium is the initial barrier to pathogenic invasion [44]. The tight junction proteins bind epithelial cells together and ensure that harmful microorganisms and toxins are not translocated into the systemic circulation [45]. The loss of intestinal integrity allows the entry of pathogens into the epithelial barrier, which causes inflammatory reactions and leads to decreased nutrient absorption rates [46]. *Eimeria* infection is a severe disorder, which leads to severe alterations in the intestinal structure by causing the death of epithelial cells throughout the intracellular parasite life cycle [47]. The effect of this damage is haemorrhage, retarded uptake of nutrients, and decreased feed efficiency [48].

Besides structural protection, the gut microbiota is critical in the protection of the host against pathogenic organisms [49]. Various microbial communities also live in the poultry intestinal tract and play a role in digestion, vitamin production, and immunity [8]. The useful microorganisms like *Lactobacillus* and *Bifidobacterium* generate organic acids and antimicrobial substances that suppress the development of detrimental microorganisms [50]. Competitive exclusion is also a process where these microbes and pathogens compete for nutrients and

points of attachment to intestinal epithelial surfaces [51]. Another necessary element of disease resistance is the interaction of gut microbiota and host immune system [52]. The microbial metabolites in the short-chain fatty acids promote the proliferation of the intestinal immune tissues and control inflammation [53]. Normal immune responses enable the host to destroy pathogens and cause minimal damage on the tissues [54]. Nevertheless, an imbalance of microorganisms, which is also known as dysbiosis, may impair immunity and predispose to infections [55].

Avian coccidiosis has been shown to significantly alter the composition of the intestinal microbiota [56]. The lysis of the epithelial cells and cellular contents provides a good environment for opportunistic pathogens [57]. Such a microbial imbalance has the potential to worsen intestinal inflammation and increasing the severity of the disease [46]. Moreover, altered gut integrity can augment the danger of colonization by other pathogens, such as zoonotic parasites [58]. Intestinal homeostasis is thus an important aspect in proper disease management in poultry production systems [8]. Nutritional therapies to promote gut health have become more popular as an alternative to conventional antimicrobial agents [59,60]. Dietary prebiotic supplementation is one such strategy, and it has proven to be promising in tuning the gut microbiota and improve host defence programs [61].

Prebiotics facilitate the growth of beneficial microorganisms and enhance the generation of metabolites that enhance the functioning of the intestinal barrier [35]. A better microbial diversity can help to restore the intestinal balance after pathogen-induced imbalance [62]. Moreover, the better mucosal immunity enables birds to get a better response against the infection by parasites [63]. Incorporation of gut health management into poultry

nutrition is one of the basic elements of sustainable disease control measures [64]. Increasing the intestinal ecosystem allows chicken farmers to decrease the occurrence and severity of such infections as coccidiosis and enhances productivity and animal welfare at the same time [13].

PREBIOTICS AS FUNCTIONAL FEED ADDITIVES

Prebiotics are non-digestible food substances that choose to stimulate the growth and metabolic activity of favourable microorganisms in the gastrointestinal tract [23]. Contrary to probiotics, which insert live microorganisms into the host, prebiotics act as food that helps to promote the growth of already existing favourable microorganisms in the gut ecosystem (Fig. 1) [65]. Prebiotics are being considered as a functional feed additive in the poultry nutrition field with the aim of improving the gut health, immune system, and resistance against infectious diseases [66,67]. Fructo-oligosaccharides, mannan-oligosaccharides, inulin, and β -glucans are common prebiotic compounds used in the diets of poultry [24]. These substances are not digested by the host enzymes in the upper gastrointestinal tract and appear in the lower intestine intact, where they undergo fermentation by useful microbial populations [68]. The end product of fermentation is short-chain fatty acids like acetate, propionate, and butyrate, which have significant functions in ensuring intestinal integrity as well as immunological responses [69].

The protective effects of prebiotics might include the development of microbial communities that prevent the colonization of pathogens, the reinforcement of intestinal tone, and the improvement of the immune response of the mucosa [70]. All these effects have the potential to

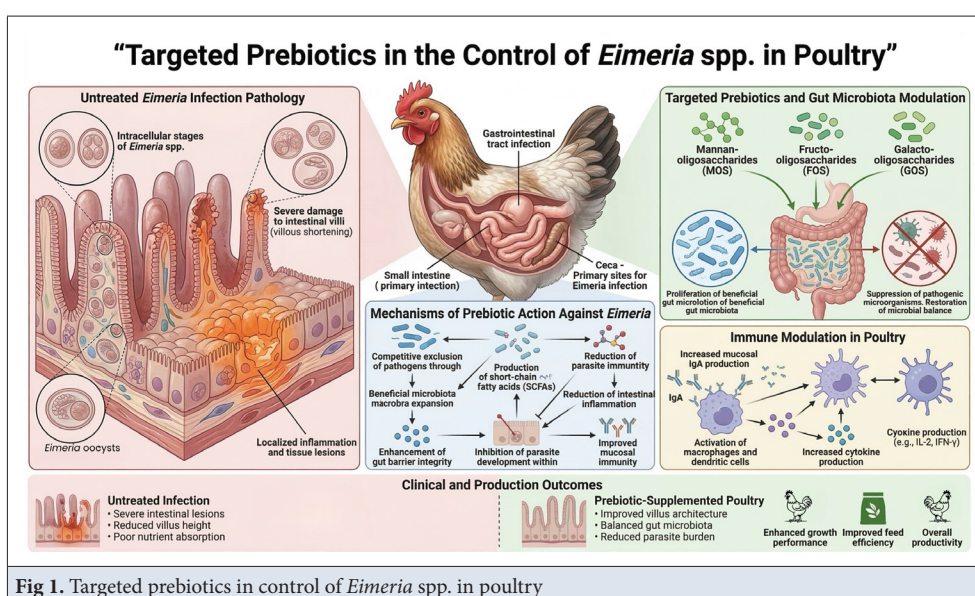


Fig 1. Targeted prebiotics in control of *Eimeria* spp. in poultry

decrease the severity of *Eimeria*-induced infections and enhance the overall performance of poultry ^[71].

Fructo-Oligosaccharides

Fructo-oligosaccharides (FOS) are a short chain fructose polymers, which are usually obtained by the use of plants like chicory, onion, and garlic ^[72,73]. The prebiotic properties of these compounds make them a popular choice as feed additives in the poultry feed industry for the selection of beneficial intestinal microorganisms ^[74]. Because of their resistance to digestion by the host enzymes in the proximal gastrointestinal tract, FOS are transported to the lower intestine unfermented to be used by the microbes ^[68]. In chickens, FOS supplementation has been shown to stimulate the growth of good bacteria, especially *Lactobacillus* and *Bifidobacterium* ^[75]. FOS fermentation by these bacteria leads to short-chain fatty acids, of acetate, propionate, and butyrate ^[76]. These metabolites are important in intestinal homeostasis because they reduce intestinal pH, prevent proliferation of pathogenic microorganisms, and enhance the integrity of epithelial cells ^[43]. Low intestinal pH will lead to an unfriendly condition for pathogenic microbes and indirectly restrict the growth of parasites like *Eimeria* ^[77].

Immunomodulatory effects, which also have anticoccidial effects, are also exerted by FOS ^[78]. Research has shown that FOS supplementation may cause an improvement in mucosal immunity by increasing the generation of immunoglobulin A ^[79] and controlling the response of cytokines in the intestinal mucosa. Improved immune reactions make the recognition and killing of the stages of intracellular parasites easier ^[58]. Also, FOS can enhance intestinal barrier operation by inducing cell regeneration of epithelial cells and preserving tight junction integrity, which restricts the invasion of parasites and tissue injury ^[43].

Another important mechanism of FOS is the control of intestinal microbiota composition ^[80]. FOS play a role in the competitive exclusion mechanism by supporting the growth of advantageous microorganisms in the small intestine, which compete with pathogens in the intestinal environment to obtain nutrients and points of attachment, which inhibits opportunistic infections commonly related to coccidiosis ^[51]. Enhanced microbial balance can also decrease secondary infections which intensify the intestinal inflammation in cases of coccidiosis outbreak ^[81]. In combination, microbiota regulation, immune-stimulation, and increased intestinal barrier integrity FOS have the potential to be a valuable nutritional solution to prevent coccidiosis in poultry and intestinal health improvement ^[82].

Mannan-Oligosaccharides

Mannan-oligosaccharides (MOS) are prebiotic compounds

derived primarily from the cell walls of yeast species such as *Saccharomyces cerevisiae* ^[83]. These oligosaccharides consist mainly of mannose residues that possess unique biological properties beneficial for intestinal health and disease resistance in poultry ^[84]. One of the most important anticoccidial mechanisms of MOS involves pathogen binding and competitive exclusion ^[39]. Many pathogenic microorganisms possess mannose-specific lectins on their surface that enable them to attach to intestinal epithelial cells ^[85]. MOS molecules mimic these binding sites and act as decoy receptors, preventing pathogens from attaching to the intestinal mucosa ^[86]. Although *Eimeria* parasites invade epithelial cells through complex host-parasite interactions, MOS supplementation may reduce the severity of infection by limiting the colonization of secondary bacterial pathogens that exacerbate intestinal damage during coccidiosis caused by *Eimeria* ^[29]. MOS also stimulates the development of beneficial gut microbial populations, including *Lactobacillus* species ^[87]. Increased populations of beneficial microbes enhance fermentation processes that generate short-chain fatty acids, improving intestinal epithelial health and promoting mucosal immunity ^[87]. Improved intestinal microbial balance reduces inflammation and supports faster recovery from epithelial damage induced by coccidiosis ^[28].

Another important mechanism of MOS involves immunomodulation ^[88]. Research has demonstrated that MOS supplementation can stimulate both innate and adaptive immune responses in poultry ^[75]. Activation of macrophages, increased production of cytokines, and enhanced antibody responses contribute to improved resistance against parasitic infections ^[89]. Additionally, MOS may stimulate the development of gut-associated lymphoid tissues, strengthening mucosal immune defences ^[90]. MOS have also been associated with improvements in intestinal morphology ^[91]. Increased villus height and enhanced absorptive surface area contribute to improved nutrient uptake and faster recovery of intestinal tissues following coccidial damage ^[92]. By promoting intestinal integrity and immune competence, MOS supplementation may significantly reduce the clinical severity and production losses associated with coccidiosis in poultry ^[93].

The integration of prebiotics into poultry feeding programs therefore represents a promising approach for enhancing productivity while simultaneously supporting animal welfare and sustainable production practices ^[94].

Inulin

Inulin is a polysaccharide that occurs naturally and is mainly composed of β -(2 $\rightarrow$ 1) linked fructose units with a glucose residue at its end ^[95]. It is often isolated out of plants like chicory root and Jerusalem artichoke and is commonly known to have prebiotic effects in

the nutrition of animals ^[96]. Since inulin is not digested by upper gastrointestinal tract enzymes, it will come to the large intestine where it is fermented by the good population of microbes ^[97]. In birds, intestinal inulin in the diet enhances the development of desirable intestinal microbes like *Bifidobacterium* and *Lactobacillus* ^[35]. Inulin fermentation results in the generation of short-chain fatty acids, which help to keep the intestinal pH at the optimum level and epithelial cell health ^[98]. One of the major products of fermentation is butyrate, which is a key source of energy to intestinal epithelial cells and is essential in the preservation of mucosal integrity ^[43].

It is presumed that the anticoccidial activity of inulin is triggered by a number of complementary mechanisms ^[12]. Inulin helps in competitive mechanisms of exclusion that prevent colonization by pathogenic microorganisms because it enhances the presence of advantageous microbial communities ^[51]. This microbial balance decreases gut inflammation, and it also contributes to a stable gut condition in *Eimeria*-induced infection ^[47].

The fermentation product of inulin improves intestinal barrier performance by increasing the growth rate of epithelial cells and strengthening tight junction proteins ^[98]. Inulin has been demonstrated to mediate the effects of the immune system by enhancing the activities of immune cells in the intestinal mucosa ^[99]. The increased production of cytokines and the improvement of secretory IgA levels augment the host's resistance to invading pathogens ^[100]. Besides exerting a direct effect on gut microbiota and immunity, inulin supplementation can also have an anti-oxidative stress and anti-inflammatory effect on intestinal infections ^[101]. Enhanced antioxidant status helps in tissue repair and healing after the damage of epithelial cells by parasitic infections ^[102]. These mechanisms will lead to better intestinal health, and inulin may be an effective dietary intervention during the prevention of the effects of coccidiosis in poultry production systems ^[5].

β-Glucans

β-Glucans are polysaccharides made of glucose molecules connected mostly by β-(1→3) and β-(1→6) glycosidic bonds ^[103]. They are usually originated through yeast, fungus, cereal, and some bacteria, and have been well known in the immunomodulatory effects on animal nutrition ^[104]. The use of β-glucans in poultry has also been widely explored as a means of boosting immune function and resistance of the animal to infectious diseases ^[105]. The main action by which the β-glucans have an anticoccidial effect is through the activation of the host immune system, where the β-glucans bind to the pattern recognition receptors (DRRs) like Dectin-1 and complement receptor 3 on the immune cells like the macrophages and dendritic cells ^[106]. Interaction of β-glucans to these receptors

causes a cascade effect of immune response that improves the host capacity to identify and exclude the invading pathogens ^[105]. This immune stimulation can enhance immunity against such parasites as *Eimeria* ^[39].

β-Glucans also provoke the synthesis of cytokines that manage inflammatory reactions and facilitating communication among the immune system cells ^[107]. There is better clearance of infected epithelial cells and parasite stages with increased activation of macrophages and natural killer cells in coccidiosis ^[39]. In addition, the β-glucans have been demonstrated to boost antibody and the growth of gut-associated lymphoid tissue ^[108]. The other positive impact of β-glucans is that it enhances the intestinal barrier integrity ^[109]. β-glucans stimulate growth of epithelial cells and facilitate processes of tissue repair that restore the intestinal architecture after being destroyed by parasitic invasion ^[5]. Mucosal integrity is improved, which minimizes the occurrence of secondary infections and increases the recovery of intestinal functions ^[44].

β-Glucans can also modify intestinal microbiota composition and make beneficial microbial populations, promoting overall gut health ^[110]. Immune stimulation, intestinal healing, and microbiota regulation have their combined effects that render β-glucans good nutritional interventions to curb coccidiosis in poultry production systems. MOS and β-Glucans can be used for direct immune boosting and pathogen blocking, while FOS and Inulin can be used to build a strong, parasite-resistant gut microbiome.

IMPACT OF PREBIOTICS ON POULTRY PERFORMANCE

The addition of prebiotics to poultry diets has been largely correlated with an increase in growth performance, feed efficiency, and overall health condition ^[35]. It is mostly credited to improved gut health, nutrient utilization, and resistance to infectious diseases ^[8]. Among the most significant benefits of prebiotic supplementation, the increased feed ratio should be mentioned ^[111]. Prebiotics promote the growth of positive intestinal microorganisms, including *Lactobacillus*, to enhance the digestion process and the breakdown of complex nutrients into absorbable forms ^[112]. Prebiotic compounds are fermented to form short-chain fatty acids that trigger the growth of the intestinal epithelial and improve gut absorptive capacity ^[113].

Another factor that is significant in determining improved poultry performance is improved intestinal morphology ^[114]. Research has shown that birds fed prebiotic supplements tend to have a higher villus height and lesser depth of the crypt in the intestinal mucosa ^[115, 116]. These enhancement measures raise the amount of surface area used in nutrient taking leading to high growth rates and high

feed utilization ^[117]. Prebiotics can also work to lessen the adverse effects of intestinal pathogens on poultry production ^[118]. *Eimeria* infections may dramatically affect growth performance because of intestinal damage, decreased feeding levels, and decreased nutrient uptake ^[119]. Prebiotics can make coccidiosis milder and help the intestinal tissues to recover faster by improving intestinal microbial balance and improving immune responses ^[120].

Besides strengthening the resistance to coccidiosis, gut health can improve the resistance to other pathogens that affect the productivity of poultry ^[93]. Enhanced mucosal immunity and the balance of microbes are useful for a stable intestinal environment, preventing the colonization of pathogens ^[121]. Consequently, the mortality rate and the general health of the flock are significantly improved in birds that were supplemented with prebiotics (*Table 1*) ^[122]. Prebiotics can also lead to a higher quality carcass and safety of the product ^[123]. Improved intestinal health results in less shedding of pathogens and the

contamination of the environment in poultry production systems ^[124]. This can reduce the risk of zoonotic pathogen contamination, like that of *Toxoplasma gondii* and *Cryptosporidium parvum* hence enhancing food safety and ensuring the health of the people ^[125,126].

FUTURE PROSPECTS

The rising research in microbiome-based disease control approaches has presented novel prospects for the field of creating specific prebiotic interventions in poultry production. The current development of high-throughput sequencing tools and microbial ecology allows gaining more information about the intricate interplay of dietary ingredients, gut microbiota, host immunity, and parasitic infection. Such technologies can help identify certain groups of microbes that lead to resilience to *Eimeria*-induced infections. Future studies can involve the development of precision prebiotics that can specifically target populations of microbes that help to boost the immunity of the host

Table 1. Summary of experimental studies evaluating the effects of dietary prebiotics on poultry gut health, immune responses, growth performance, and control of coccidiosis caused by *Eimeria* species

Sr. No.	Prebiotic	Source	Poultry Type	Experimental Design	Dose / Inclusion	Duration	Target Condition	Parameters Evaluated	Mechanism	Results	Ref.
1	Mannan-oligosaccharides (MOS)	Yeast cell wall from <i>Saccharomyces cerevisiae</i>	Broiler chickens	Experimental infection with coccidia	MOS supplemented diet	3-4 weeks	Infection with <i>Eimeria tenella</i>	Oocyst shedding, caecal lesion score	Enhanced immune response and gut protection	Reduced number of schizonts in infected birds	[127]
2	MOS + probiotic synbiotic	Yeast-derived MOS + bacterial culture	Hubbard broilers	Controlled feeding experiment (250 chicks)	Prebiotic supplementation with probiotic combination	Starter to finisher phase	Growth performance & immunity	BW gain, FCR, antibody titers, intestinal morphology	Gut microbiota modulation and immune activation	Improved bodyweight gain, villus height, and antibody levels	[128]
3	MOS and FOS	Yeast-derived MOS and plant FOS	Broiler chickens	Feeding trial (240 chicks)	0.1-0.2% in diet	6 weeks	Growth and meat quality	Carcass traits, lipid oxidation, cholesterol content	Improved nutrient absorption and microbial balance	Improved carcass yield and antioxidant stability	[129]
4	Fructo-oligosaccharides	Plant-derived oligosaccharides	Cobb 500 broilers	Two production cycles feeding experiment	5 g/kg feed	5 weeks	Immune response & microbial profile	Immune cell response, microbial counts	Lower intestinal pH and stimulation of beneficial microbes	Higher cellular immune response and reduced pathogenic bacteria	[130]
5	MOS prebiotic	Yeast cell wall polysaccharide	Commercial broilers	Controlled feeding trial	Dietary MOS supplementation	42 days	Growth and gut histology	BW gain, FCR, gut morphology	Enhanced villus height and nutrient absorption	Improved intestinal morphology and performance	[131]
6	MOS, FOS, and inulin	Multiple prebiotic sources	Ross 308 broilers	Randomized feeding experiment	Dietary supplementation	42 days	Metabolism & gut microbiota	Protein digestibility, gut microbial composition	Fermentation producing beneficial SCFA	Improved gut microbial diversity and nutrient metabolism	[132]
7	FOS and MOS	Plant and yeast oligosaccharides	Broiler chickens	Controlled feeding experiment	5g/kg each	5 weeks	Microbial balance	LAB count, pathogenic bacteria count	Competitive exclusion and acidic gut environment	Reduced <i>E. coli</i> and improved microbial balance	[133]
8	Glucomannan (prebiotic fiber)	Plant tuber (<i>Amorphophallus muelleri</i>)	Broiler chickens	Feeding experiment	0.25% diet	Broiler growth cycle	Gut microbiota & growth performance	BW gain, FCR, microbiome sequencing	Promotion of beneficial microbes such as <i>Lactobacillus</i> spp.	Improved microbial diversity and feed efficiency	[134]
9	MOS + FOS	Prebiotic mixture	Broilers	Comparative study vs antibiotic growth promoters	0.1-0.2% diet	42 days	Meat quality & antioxidant status	Lipid oxidation, meat cholesterol	Improved antioxidant status and microbiota	Reduced cholesterol and oxidative damage	[129]
10	Prebiotic supplementation under stress	MOS-based prebiotic	Broiler chickens	Heat-stress experimental trial	Dietary supplementation	Several weeks	Heat stress and immune suppression	BW, mortality, WBC count	Improved gut microbiome and immune defence	Improved feed intake and reduced mortality	[122]

and inhibit the growth of pathogenic microbes. Although these nutritional techniques can improve disease control and poultry performance, combining prebiotics with other nutritional measures like probiotics, phytochemicals, and vaccines could enhance the results of this effort. The alternative potential research area is the study of synbiotic preparations involving the combination of prebiotics and useful microorganisms. Such solutions can be found to have a synergistic effect to enhance intestinal health and resistance to infectious diseases. Also, long-term field experiments are required to check the efficacy of these strategies under commercial conditions of poultry production. Further investigation of microbiome-based nutrition interventions can eventually lead to poultry production systems that are sustainable and free of antibiotics.

CONCLUSION

Avian coccidiosis has continued to be one of the greatest problems in poultry production, mainly because of infections by protozoan parasites of *Eimeria* genus. Conventional methods of control involving anticoccidial medication and vaccination have been effective in the management of the disease over many years but the development of drug-resistant strains of parasites and increasing limitation on the use of antimicrobial agents have led to the need to seek alternative control methods. Specific prebiotics are a novel nutritional solution to enhance intestinal condition and increase coccidiosis resistance. Fructo-oligosaccharides, mannan-oligosaccharides, inulin, and β -glucans have a positive influence through various mechanisms that include the regulation of gut microbiota, an increase in mucosal immunity, and an increase in intestinal barrier integrity. These effects lower the severity of coccidiosis but also lead to an increase in growth performance and feed efficiency in poultry. Moreover, better gut health can lead to an increased resistance to concomitant parasite zoonotic pathogens (including *Toxoplasma gondii* and *Cryptosporidium parvum*), which can improve food safety and the health of the population. Further studies on microbiome-specific oriented nutritional interventions can offer useful options for the management of poultry diseases and the design of production systems without antibiotics.

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RESEARCH ARTICLE

Pre-Administration with *Nigella sativa* Seed Powder Caused Alterations in Antibody and Serum Interferon Gamma Profiles in Cockerels Challenged with a Very Virulent Infectious Bursal Disease Virus

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Abstract

Infectious bursal disease (IBD) compromises the immune system of young birds, lowering protective antibody levels. This study assessed antibody and serum interferon-gamma (INF- γ) responses in cockerels pre-treated with *Nigella sativa* seed powder (NSSP) and challenged with very virulent IBD virus (vvIBDV). One hundred one-day-old Dominant Black Marshal Cockerels were assigned to five groups (A-E, 20 birds each). Groups A and B received standard feed from 1-42 days of age; groups C and D received NSSP from 21-27 days of age; group E received NSSP continuously from 1-42 days of age. All birds were vaccinated against Newcastle disease. On day 28, groups B, D, and E were orally challenged with vvIBDV. Blood samples were analyzed for IBD antibody and INF- γ . Maternal antibody titres declined below protective levels by 14 days in groups A-D but remained protective in group E. Post-challenge, groups D and E showed significantly higher antibody titres, with group E maintaining the highest levels. Serum INF- γ increased from 7 to 21 days of age in all groups, and from 29 to 42 days of age, group E exhibited the highest levels compared to D and B. Pre-administration of NSSP slowed maternal antibody decay and enhanced post-challenge immune responses. Dietary inclusion of NSSP may therefore provide a natural strategy to sustain IBD antibody protection and improve immune performance in poultry.

Keywords: Antibody, Infectious bursal disease, Interferon gamma, *Nigella sativa*

INTRODUCTION

Poultry health is a critical component of global food security and agricultural sustainability, with infectious diseases posing substantial economic and welfare challenges. One of the most devastating diseases affecting young chickens is infectious bursal disease (IBD), an acute and highly contagious viral infection caused by the infectious bursal disease virus (IBDV), particularly its very virulent strains (vvIBDV) ^[1,2]. Infectious bursal disease primarily targets the bursa of Fabricius leading to immunosuppression and increased susceptibility to secondary infections or vaccine failures ^[3,4]. The decay of maternally derived antibodies (MAB) over time without

adequate immunological response has been identified as a major vulnerability point in early chick development ^[5]. In addition to antibody-mediated protection, cell-mediated immunity plays a vital role in the host's defense against IBDV infection. Serum interferon-gamma (INF- γ), a key cytokine involved in antiviral and immunomodulatory activities, serves as an indicator of immune activation and resistance in infected or immunostimulated birds ^[6,7].

Conventional vaccination programmes remain the mainstay for IBD prevention; however, maternal antibody interference and the timing of vaccination present persistent limitations. As maternal antibodies wane, typically within the first two to three weeks of life, chicks become increasingly vulnerable to vvIBDV infections ^[5,8].



Therefore, understanding and managing the dynamics of maternal antibody decay is essential to optimize vaccination efficacy and ensuring robust immune protection. Also, the use of adjunct strategies that can stabilize or extend protective antibody levels during this critical window are needed to supplement conventional immunization protocols.

Nigella sativa (black seed), a traditional medicinal plant widely studied for its antioxidant, anti-inflammatory, and immunomodulatory properties, has gained attention in veterinary immunonutrition^[9-11]. Bioactive compounds in *N. sativa*, particularly thymoquinone, have been shown to modulate immune responses and improve antibody production in various animal models^[12,13]. These immunomodulatory effects have been demonstrated across a range of disease conditions, including viral infections such as Newcastle disease and infectious bursal disease, bacterial infections, and inflammatory disorders. In poultry, *N. sativa* supplementation has been associated with enhanced humoral immune responses following vaccination and experimental infections, suggesting its potential role in improving resistance to immunosuppressive diseases such as IBD. Thymoquinone, the major bioactive component, is believed to enhance B-cell activity, cytokine production, and antioxidant defense mechanisms, thereby supporting both humoral and cell-mediated immunity^[14-16].

This study was designed to evaluate the impact of pre-administration with *N. sativa* seed powder (NSSP) on the maternal antibody decay and the antibody response in cockerels experimentally challenged with a vvIBDV strain. The study hypothesized that continuous or strategic inclusion of NSSP in chick diets may prolong the protective duration of maternally derived antibodies and enhance post-challenge immune responses. The study aims to provide insights into the potential role of NSSP as a natural immunomodulatory agent through evaluation of the kinetics of antibody titres and serum INF- γ levels across different time points and feeding regimens. This would be significant in the design of better nutritional and health strategies for poultry, and also contribute to the growing interest in phytogenic feed additives as sustainable alternatives to synthetic drugs.

MATERIAL AND METHODS

Ethical Approval

The Ahmadu Bello University committee on Animal Use and Care (ABUCAUC) granted the ethical approval for this study with approval number ABUCAUC/2024/79.

Source of Birds

One hundred, one-day-old Dominant black marshal

cockerel (DBMC) chicks were purchased from a Hatchery (Zartech, Ibadan) and transported to the Research Pen of the Department of Veterinary Pathology, Ahmadu Bello University, Zaria.

Housing and Management of Birds

The poultry housing unit was thoroughly cleaned using water and detergent, followed by disinfection. Fumigation of the pen was carried out twice at two-week intervals prior to the arrival of the chicks. Upon their arrival, the area surrounding the pen was fumigated twice weekly with disinfectants throughout the duration of the study. The chicks were brooded on deep litter, and wood shavings were used as bedding material. Each group was provided with its own feeder and drinker. Feed and water were made available to the chicks at all times. Control of rodents and insects was achieved through the application of rodenticides and insecticides, administered twice at one-week intervals.

Source of Feed

Vital feed® chick mash was used in this study, and it was purchased from a commercial sales outlet in Zaria.

Sources of Challenge Virus and Vaccine

The Viral Research Department of National Veterinary Research Institute (NVRI), Vom, Plateau State provided the vvIBDV used. Also, the vaccines against Newcastle disease (ND): (ND La Sota) was produced by NVRI, Vom, and was purchased from a commercial sales outlet in Kaduna.

Source of *Nigella sativa* Seed Powder

Nigella sativa seeds were obtained from Herbal Point, Samaru, Zaria, Kaduna State, and was taken to the Department of Botany, Faculty of Life Sciences, A.B.U. Zaria for identification. Thereafter, the seeds were grinded into the powdered form.

Preparation of *Nigella sativa* Seed Powder and Feed Mixture

The *Nigella sativa* seed powder (NSSP) and feed mixture was prepared by mixing 2.8 g of NSSP with 1 kg of feed^[14].

Vaccination of Birds Against Newcastle Disease

All the chicks were vaccinated against ND using ND vaccine La Sota at 7 and 17 days of age.

Grouping of Birds

The chicks were randomly divided into 5 groups, A, B, C, D, and E, of 20 birds each. Birds in groups A (Negative control) and B (Positive control) were administered feed only; C (NSSP from 21 to 27 days of age) and D (NSSP from 21 to 27 days of age + vvIBDV) were administered the NSSP + feed consecutively from 21 to 27 days of age,

while E (NSSP from 1 to 42 days of age + vvIBDV) was administered the NSSP + feed consecutively for from 1 to 42 days of age. At 28 days old, only birds in groups B, D, and E were challenged with a vvIBDV orally.

Challenge of Birds with Very Virulent Infectious Bursal Disease Virus

Each bird was challenged by oral administration of 0.2 mL of vvIBDV suspension with virus titre of $10^{8.50}$ CID₅₀/mL.

Collection of Blood

Blood was collected on 1, 7, 14, 21, 28, 29, 30, 31, 32, 33, 34, 35, 38, and 42 days of age from all chickens via the brachial vein and serum was harvested. The serum was assayed for antibodies against IBDV and serum INF- γ using enzyme linked immunosorbent assay (ELISA).

Enzyme Linked Immunosorbent Assays

ELISA was used to detect IBDV antibody and INF- γ in the sera of the cockerels using IBDV antibody test kit (ID Screen® IBD VP2 - ID.vet Innovative Diagnostics, France) and chicken INF- γ ELISA kit (Pioway Medical Lab Equipment Co., Ltd. Nanjing, China), respectively by following the manufacturers' instructions. The optical density (OD) values at 450 nm wavelength were measured using ELISA microtitre plate reader (Thermo Scientific, Multiskan Ex). Thereafter, the absolute level of antibody and INF- γ in the sample was calculated according to the manufacturer's recommendation. For the IBD antibody, the breakthrough titre was 845, and antibody titre ≥ 845 was considered protective, while titre < 845 was considered not protective.

Histopathological evaluation of the Bursa of Fabricius was not included in this study, as the focus was primarily on functional immune responses (antibody titres and serum IFN- γ levels).

Data Analysis

Chart was used for data presentation. Data was expressed as mean $\pm$ SEM and subjected to repeated measure One-way analysis of variance (One-way ANOVA) followed by Turkey's *post hoc* test. Graphpad prism version 8.0 (San Diego California, USA) was used for the analysis, and values of $P \leq 0.05$ were considered significant.

RESULTS

No mortality was recorded in any of the experimental groups throughout the study period, including after challenge with vvIBDV.

Decay in Maternal Antibody

The mean IBD maternal antibody titre decreased significantly ($P < 0.05$) from protective level (at 1 days of age) to below breakthrough titre (< 845) at 14 days of

age in chickens of groups A (2067 ± 87.62 ; 726.2 ± 93.26), B (2121 ± 63.76 ; 740.6 ± 64.12), C (2107.4 ± 51.05 ; 716.9 ± 65.43) and D (2132.8 ± 97.74 ; 738.1 ± 31.99) (Fig. 1). There was a significant ($P < 0.05$) decrease in the mean IBD maternal antibody titre in groups E (2076 ± 63.98 ; 1008.6 ± 84.03), from 1 to 14 days of age, but the titres were within the protective level (> 845). At 21 days of age, the mean IBD maternal antibody titre increased in all groups, but this was significantly ($P < 0.05$) higher and within protective level in group E (1036 ± 61.84) compared to groups A (815.9 ± 46.83), B (797.2 ± 73.67), C (786.7 ± 49.14), and D (786.4 ± 42.56). At 28 days of age, the mean IBD maternal antibody titre was significantly ($P < 0.05$) higher and within protective level in chickens of group E (975.4 ± 47.63) compared to A (576.7 ± 93.26), B (594.6 ± 64.12), C (651.3 ± 51.15), and D (675.3 ± 39.26) (Fig. 1).

Antibody Response Post-Challenge

From 28 to 42 days of age, there was a significant ($P < 0.05$) decrease in the mean IBD antibody titre in groups A (576.7 ± 93.26 ; 85.25 ± 14.74) and C (651.3 ± 51.15 ; 60 ± 9.14) (Fig. 1). The mean antibody titre increased ($P < 0.05$) significantly from below protective level (from 28 to 31 days of age) to protective level (from 32 to 38 days of age), and followed by a decrease within protective level (at 42 days of age) in group B (594.6 ± 64.12 ; 1294 ± 73.29 ; 917 ± 44.18). In groups D (675.3 ± 39.26 ; 1585 ± 48.76 ; 1361 ± 69.95) and E (975.4 ± 47.63 ; 2118 ± 31.45 ; 1976 ± 61.63), the mean antibody titre increased ($P < 0.05$) significantly from 28 to 38 days of age, followed by a decrease within protective level at 42 days of age, but the titre was significantly ($P < 0.05$) higher in group E compared to D (Fig. 1).

Changes in Serum Interferon Gamma Level

The mean ($\pm$ SEM) serum interferon gamma (INF- γ) level showed no significant ($P > 0.05$) difference in all the

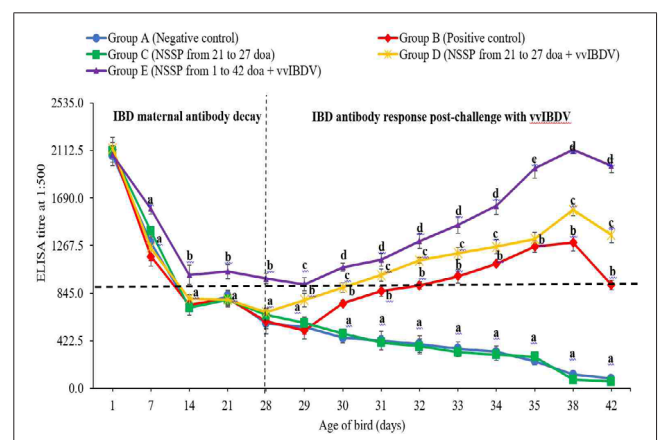


Fig 1. Infectious bursal disease maternal antibody profile of chickens administered *Nigella sativa* seed powder and challenged with a very virulent infectious bursal disease virus at 28 days of age. Values with different alphabets in the same day differ significantly at $P < 0.05$. NSSP-*Nigella sativa* seed powder; doa-days of age

groups of chickens from 1 to 28 days of age, but was non-significantly ($P>0.05$) higher in group E at 7, 14, 21, and 28 days of age. In all the groups, the mean serum INF- γ level increased ($P<0.05$) significantly from 7 to 21 days of age, followed by a decrease at 28 days of age, but with no significant difference between the groups (Fig. 2).

From 29 to 35 days of age, the mean serum INF- γ level increased significantly ($P<0.05$) in groups B, D and E, but was significantly ($P<0.05$) higher in group E (19.76 ± 5.72 ; 88.92 ± 5.65 pg/mL) followed by group D (21.27 ± 2.95 ; 63.32 ± 4.24 pg/mL) compared to group B (9.76 ± 4.83 ; 51.41 ± 4.60 pg/mL), A (5.68 ± 0.95 ; 2.93 ± 0.75 pg/mL) and C (7.14 ± 2.83 ; 3.91 ± 0.89 pg/mL). This was followed by a decrease in the mean serum INF- γ level, at 38 and 42 days of age, but with a significantly ($P<0.05$) higher value in group E (78.26 ± 4.53 ; 70.68 ± 7.95 pg/mL) followed by group D (58.05 ± 3.65 ; 42.98 ± 3.18 pg/mL) compared to group B (47.53 ± 4.13 ; 36.38 ± 5.66 pg/mL), A (1.27 ± 0.57 ; 1.74 ± 0.65 pg/mL) and C (2.74 ± 0.92 ; 2.20 ± 1.09 pg/mL) (Fig. 2).

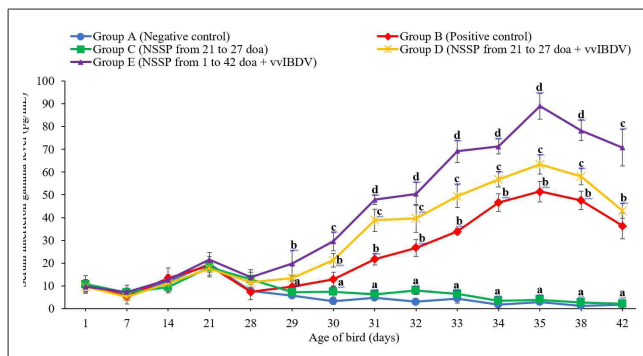


Fig 2. Mean ($\pm$ SEM) serum interferon gamma level in chickens administered *Nigella sativa* seed powder and challenged with a very virulent infectious bursal disease virus at 28 days of age. Values with different alphabets on the same day differ significantly at $P<0.05$. NSSP-*Nigella sativa* seed powder; doa-days of age

DISCUSSION

The significant decrease in mean IBD maternal antibody (MAB) in all the groups of chickens, from 1 to 14 days of age in this study is consistent with previous reports [8,17]; therefore, the eventual decline may support the need for timely vaccination strategies to enhance immunity as maternal protection wanes. This decrease over time could be associated with the spontaneous breakdown of MAB [5,18]. The decrease below breakthrough titre at 14 days of age in groups A, B, C, and D might suggest a loss of passive immunity provided by MAB. This decline is vital as it may indicate the high susceptibility of young chicks to IBD during this period, since existing literature has highlighted the role of MAB in providing initial protection against IBD in chickens [5,19].

Moreover, the MAB titres in group E remained at protective levels (compared to groups A and B) until 28 days of age. This may suggest that NSSP administration may delay MAB decay, possibly by preserving immune integrity through its antioxidant and anti-inflammatory effects. Possible mechanism for this decreased MAB decay might be linked to direct or indirect interference with the MAB breakdown processes, by bioactive compounds, such as thymoquinone in NSSP. However, in groups C and D, the MAB levels likely declined more rapidly compared to group E, and this could be associated with the duration of NSSP administration as previously suggested. Hence, this prolonged NSSP administration might have helped to maintain higher levels of MAB for a longer duration, thereby providing better protection against the decay of these antibodies. This may be due to the fact that the continuous presence of bioactive compounds of *N. sativa* might have enhanced the stability and longevity of MAB, and/or decreased their breakdown, thus, reducing their rate of decay. This phenomenon aligns with previous studies that indicated that dietary antioxidants from plants can support immune functions and prolong the effectiveness of MAB in poultry [20-23].

Post-challenge with vvIBDV, there was an increased IBD antibody titre in groups B, D, and E, and this is consistent with previous studies that reported increased IBD antibodies following challenge with IBDV in chickens [8,17,24]. Chickens in group B showed a significant increase in antibody titres from below breakthrough titre to protective levels between 32 to 38 days of age. This may indicate an effective immune response following viral exposure despite initial low titres. In contrast, groups D and E also demonstrated increased antibody titres but maintained significantly higher levels compared to group B throughout the observation period. This finding suggests that NSSP might have played a role in the enhancement of immune responses post-challenge, probably through its immunomodulatory properties [23], as previously suggested. This is supported by the observed quantitative differences, where chickens in group E consistently maintained significantly higher antibody titres compared to other challenged groups, remaining well above the protective threshold (>845) for a longer duration. However, the higher antibody response in group E compared to group D may be associated with a more effective priming of the immune system, and/or enhanced activation and proliferation of B cells, resulting from the sustained administration of NSSP. In addition, the limited administration of NSSP in group D might have resulted in a less effective immune activation following the vvIBDV challenge.

The serum INF- γ levels, from 1 to 28 days of age, suggests that the baseline immune status was similar among the

groups prior to the vvIBDV challenge. The increase in INF- γ levels from 7 to 21 days of age across all groups indicates an initial immune activation, likely as a result of vaccination against Newcastle disease (ND) at 7 and 17 days of age, which is known to stimulate cellular immunity and enhance the overall immune response. Following the vvIBDV challenge, groups B, D, and E demonstrated significant increases in serum INF- γ levels from 29 to 35 days of age, with group E showing the highest levels. This elevation in INF- γ may indicate a robust Th1-type immune response, which is crucial for combating viral infections. In the present study, this was evident from the significantly higher INF- γ concentrations recorded in NSSP-treated groups, particularly group E, during the post-challenge period, indicating enhanced cellular immune activation. The significantly higher INF- γ levels in group E suggest that pre-administration of NSSP might have enhanced the immune response by promoting lymphocyte activation and proliferation. Previous studies have shown that *N. sativa* possesses immunomodulatory properties that could enhance cytokine production, thereby improving ability of the host to respond to infections^[25,26].

At later time points (38 and 42 days of age) in this study, group E continued to show significantly higher INF- γ levels compared to other groups, and this may indicate sustained immune activation. This prolonged elevation may be attributed to the combined effects of *N. sativa* and prior vaccination against ND, which may have primed the immune system for a more effective response against vvIBDV. This suggests that the immune system was more effectively primed due to continuous dietary supplementation and initial ND vaccination, thus, led to a quicker and stronger antibody production in response to vvIBDV challenge. However, this assertion requires further investigation, and it highlights the importance of both vaccination and dietary supplementation in enhancing immune responses in poultry. Although the findings demonstrate clear immunomodulatory effects of NSSP, further investigations incorporating histopathological assessment of lymphoid organs (especially the Bursa of Fabricius), viral load quantification, and broader cytokine profiling would provide deeper insight into the mechanisms underlying these observations.

Pre-administration with *N. sativa* seed powder decreased the maternal antibody decay, and enhanced the antibody response in cockerels challenged with a vvIBDV. Hence, dietary inclusion of NSSP may constitute a natural strategy to sustain protective IBD maternal antibody levels and improve immune response in poultry production. However, further studies integrating pathological and molecular assessments are recommended to fully elucidate the mechanisms involved.

DECLARATION

Availability of Data and Materials: Data and materials for this research are available upon request.

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Ethical Approval: The Ahmadu Bello University committee on Animal Use and Care (ABUCAUC) granted the ethical approval for this study with approval number ABUCAUC/2024/79.

Conflict of Interest: The authors declared 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.

Author Contributions: SBO, BM and IWM contributed to the design of this study. MSM, OO, and MOE participated in the sample collection, data analysis. OO wrote the original draft. All authors contributed to data collection and discussion.

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RESEARCH ARTICLE

Molecular Evidence, Phylogenetic Evaluation, and Antibigram Profiling of Methicillin-Resistant *Staphylococcus aureus* Isolated from Respiratory Tract Infections of Cats in Pakistan

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Abstract

Antimicrobial-resistant *Staphylococcus aureus* particularly methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide challenge for both veterinary and human health. This study investigated MRSA in respiratory tract infections of cats, associated risk factors, antibiogram profiling and synergy testing for resistance modulation. A total of 103 nasal swabs collected from cats were processed to identify and confirm *S. aureus* and MRSA using microbiological, biochemical, and molecular tests. Furthermore, antibiogram profiling and in-vitro trials for resistance modulation against MRSA were also conducted. The molecular prevalence of *S. aureus* in respiratory tract infections in cats was 37.86%. Among infected cats, phenotypic and genotypic prevalence of MRSA was 64.10% and 38.46%, respectively. Antibiotic susceptibility trials against MRSA revealed highest resistance against Ceftriaxone, Cefixime, and Penicillin G (100%). Resistance modulation trials revealed synergism between Ceftriaxone with Ketoprofen against MRSA. Risk factors analysis revealed significant association including respiratory illness, housing hygiene, human contact, and diagnostic resources. Phylogenetic analysis of *mecA* sequences revealed four study isolates (PP768062, PP852380, PP848319, PP848320) showing significant similarity with each other and with *mecA* sequences already reported from Myanmar and Pakistan, while one isolate (PP848321) showed significant similarity with *mecA* from Nigeria, India, Pakistan, and Iraq. The study provided useful insights into resistance profiling and resistance modulation to tackle challenge of antimicrobial resistance in *S. aureus*.

Keywords: Antimicrobial resistance, Molecular characterization, MRSA, Phylogenetic analysis, *Staphylococcus aureus*

INTRODUCTION

Antimicrobial resistance (AMR) is among the top ten global health risks to humans and animals^[1]. Antimicrobial-resistant organisms can easily transmit between the environment, humans, and animals, especially when inadequate infection control practices are coupled with a sharp rise in emerging bacterial resistance^[2]. Due to genetic flexibility, bacteria can adapt themselves to different environmental extremes, such as antibiotics that can endanger their survival, ultimately leading to the emerging and prevailing issue of AMR^[3]. Companion animals like cats and dogs, which remain in close vicinity of human beings, may act as a potential source of transmission of these antimicrobial-resistant pathogens to human beings, leading to severe health consequences^[4-6].

Among antibiotic-resistant bacteria, *Staphylococcus aureus* is a notorious pathogen due to its multidrug-resistant clones^[7] and its resistance to the majority of currently prescribed antibiotics^[8]. *S. aureus* is a gram-positive bacterium that has been incriminated in several potentially fatal illnesses in humans and animals, including pets^[9]. Community and hospital-acquired *S. aureus* infections put a significant burden in the form of healthcare costs, morbidity, and mortality^[10]. *S. aureus* is a common nasal microflora present in both immune-compromised and healthy pet animals, including cats and dogs^[11]. The habitat of *S. aureus* in pets is the anterior nares^[5], and being an opportunistic pathogen, it can worsen the respiratory tract infections of cats. Moreover, *S. aureus* has the potential to spread from companion animals to humans, therefore spreading antimicrobial resistance to other species^[12].



S. aureus sheds from mucous membranes and skin of colonized animals, contaminating the environment and eventually serving as a cause of many diseases [13].

S. aureus can develop multidrug resistance (MDR), which helps bacteria evade antimicrobial effects along with the host defense system [6,14,15]. Among antimicrobial-resistant strains of *S. aureus*, methicillin-resistant *S. aureus* (MRSA) is of high significance among veterinary and human medicine. The *mecA* gene is considered responsible for methicillin resistance, which encodes the production of the PBP2a protein. PBP2a protein has a lower affinity for beta-lactam group of antibiotics (penicillin, cephalosporins, carbapenems), which makes *S. aureus* resistant to these commonly used antimicrobials [16].

Due to potential household interspecies transmission of *S. aureus* and MRSA, it is pertinent to study the nasal carriage of these pathogens in cats. The MRSA has been documented in cattle [8], equines [13,17], buffalo [6,18], goats [4], and sheep [19] in Pakistan, but comprehensive research on MRSA in respiratory tract infections of cats to assess the likelihood of interspecies transmission is lacking in Pakistan. This study provides molecular evidence of MRSA from respiratory tract infections of cats, especially focusing on sequencing and phylogenetic analysis. The main purpose of this study was to investigate the prevalence of *S. aureus* and specifically MRSA prevalence in client-owned and cattery cats, along with the analysis of possible associated risk factors. Moreover, antibiotic susceptibility profiling of study isolates, along with possible resistance modulation strategies, were also evaluated in this study.

MATERIAL AND METHODS

Ethical Approval

The study was approved by the Advanced Studies and Research Board (ASRB), University of Veterinary and Animal Sciences, Lahore-Pakistan vide letter no. DAS/498; Dated 15.04.2024.

Study Design and Population

The sampling was done from client-owned cats brought to the veterinary clinics and cats reared in catteries based in the district Lahore, Punjab, Pakistan (Fig. 1). A total of 103 samples (n=103) consisting of client-owned cats (n=36) and cattery cats (n=67) were collected using convenient sampling technique from January 2023 to December 2023 [4]. Animals with fever, anorexia, and respiratory signs such as coughing, nasal discharge, and dyspnea were considered for sampling.

Sample Collection and Risk Factors Analysis

Nasal swabs were collected from client-owned cats and catteries following the guidelines of Shoaib et al. [20]. A

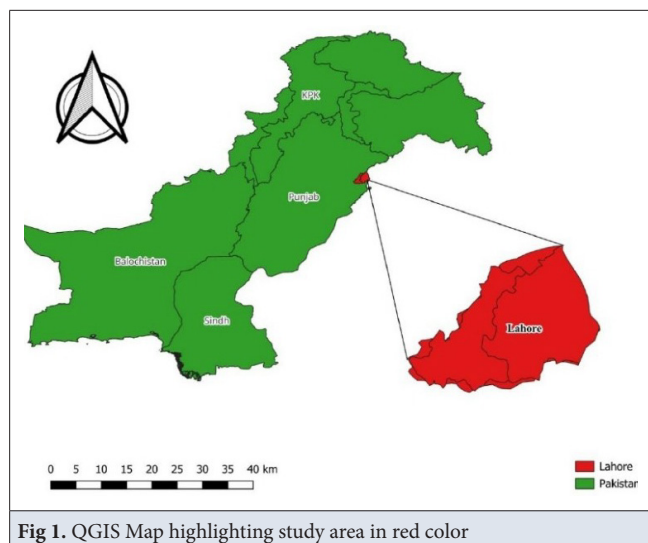


Fig 1. QGIS Map highlighting study area in red color

sterile swab dipped in Phosphate Buffered Saline (PBS) was used for sampling. A swab was inserted into the cat's nostrils and withdrawn after proper contact with the mucosa. Maintaining sterility, nasal swabs dipped in normal saline were transferred to the Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan. A predesigned questionnaire was also filled out while sampling, which included information regarding possible risk factors that can be connected with *S. aureus* and MRSA-associated respiratory tract infection of cats. Major risk factors include species, sex, age, housing type, respiratory signs, general physical condition, presence of other animal species, stocking density, antibiotic usage, outdoor access, housing hygiene, human contact, and season.

Isolation and Confirmation of *S. aureus*

First of all, the nasal swab samples were processed for enrichment by placing them in Brain Heart Infusion broth containing dextrose 2 g/L, disodium phosphate 2.5 g/L, proteose peptone 10 g/L, and NaCl 5 g/L, accompanied by an incubation at 37°C for 24 h. After enrichment, swabbing was performed on blood agar for hemolysis pattern and then culturing on selective media, MSA for *S. aureus* [13]. *S. aureus* isolates were identified based on characteristic features of colonies, fermentation of mannitol sugar present in MSA, purple color on gram staining, and positive results on coagulase and catalase tests [6,21]. For genotypic detection of *S. aureus*, DNA was isolated from phenotypically positive *S. aureus* isolates using the DNA extraction kit (Thermoscientific Gene JET Genomic DNA purification kit K0721) method [6]. The DNA samples were then stored at -20°C for subsequent analysis [18]. DNA quantification was performed by the nano-drop assay. After quantification, isolates were genotypically confirmed by PCR targeting the presence or absence of the *nuc* gene. The 270 base pair *nuc* gene

fragment was amplified using the amplification conditions and primers (**P1**: GCGATTGATGGTGATACGGTT, **P2**: AGCCAAGCCTTGACGAATAAGC) described by Louie et al.^[22] (Fig. 2).

Phenotypic Identification of Methicillin-Resistant *S. aureus*

Methicillin-resistant *S. aureus* was phenotypically identified by the Kirby-Bauer disc diffusion method using Cefoxitin discs. A cefoxitin disc was placed on *S. aureus* growth on MHA plates. Agar plates were then incubated at 37°C for 24 h. After incubation, isolates were classified as methicillin sensitive or methicillin resistant based on zones of inhibition measurements as per the guidelines of ^[23]. Isolates that showed zones of ≥ 22 mm were considered as methicillin-sensitive isolates, while isolates with inhibition zones of ≤ 21 mm were considered as methicillin-resistant isolates.

Genotypic Confirmation of Methicillin-Resistant *S. aureus*

MRSA was genotypically identified by PCR targeting the *mecA* gene. Primers used for PCR were documented by ^[24]. The primers were P1: 5'-TGGCATTTCGTGTCACAATCG-3', P2: 5'-CTGGAACCTTGTTGAGCAGAG-3' with a product size of 310 bp. The conditions of PCR are listed as: initial DNA denaturation at 94°C for 5 min, accompanied by a second step in which 34 cycles of final denaturation at 94°C for 1 min, annealing at 54°C for 1 min, initial extension at 72°C for 1 min, and then final extension at 72°C for 10 min ^[21]. The amplified PCR product was analyzed using ethidium bromide dye on a 1.5% agarose gel and observed via ultraviolet light ^[25,26] (Fig. 3).

Sequencing and Phylogenetic Analysis of the Staphylococcal *mecA* Gene

The product obtained after gel electrophoresis was purified using a gel extraction kit (Thermoscientific GeneJET). Purified gel was forwarded for sequencing to the renowned laboratory (Address: 1st BASE, JTC MedTech Hub, Tukang Singapore 618,305).

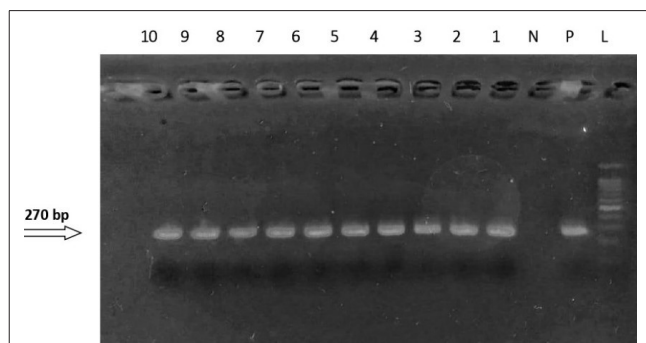


Fig 2. Amplified PCR product of *nuc* gene illuminated under UV-illuminator. L = Ladder, P = Positive control, N = Negative control, 1-10 = Sample 1 to 10

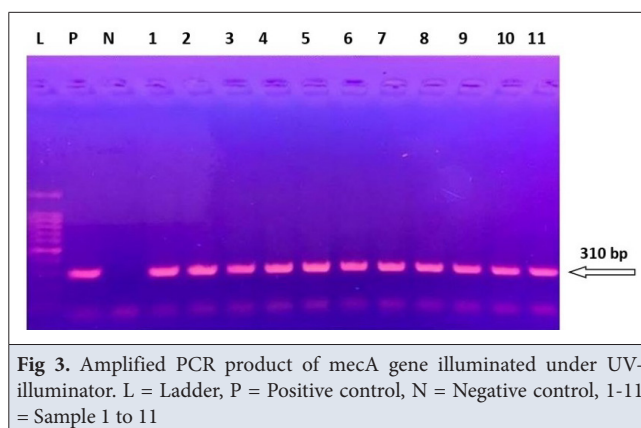


Fig 3. Amplified PCR product of *mecA* gene illuminated under UV-illuminator. L = Ladder, P = Positive control, N = Negative control, 1-11 = Sample 1 to 11

For phylogenetic analysis of sequenced isolates, sequences of the *mecA* gene reported by different countries were obtained from NCBI. Using specialized tools, including BLAST and the ClustalW method of MEGA-X (version 11.0.13), nucleotide sequences were compared and aligned. After sequence alignment, MEGA-X (version 11.0.13) was used to construct a phylogenetic tree with Maximum Likelihood method at 1000 bootstrap values ^[13].

In vitro Antibiotic Susceptibility Trials

First of all, the susceptibility of phenotypically and molecularly confirmed *mecA*-positive isolates was checked against commonly used antibiotics using the disc diffusion test and well diffusion assay. After this, susceptibility against NSAIDs (Ketoprofen and Meloxicam) alone was checked using the well diffusion assay. Finally, susceptibility against antibiotics that were highly resistant in combination with NSAIDs was assessed via the well diffusion assay to look for resistance modulation.

Susceptibility of MRSA Isolates Against Antibiotics

Antibiotic discs were purchased in order to carry out disc diffusion trials from an industry named Bioanalyse. The antibiotics include Penicillin G (10 µg), Ampicillin (10 µg), Amoxicillin (25 µg), Cephalexin (30 µg), Ceftriaxone (30 µg), Cefixime (5 µg), Vancomycin (30 µg), Cefoxitin (30 µg), Tylosin (30 µg), Linezolid (30 µg), Levofloxacin (5 µg), Oxytetracycline (30 µg), Doxycycline (30 µg), as well as Ciprofloxacin (5 µg). Muller Hinton agar was swabbed with bacterial growth (1×10^8 CFU/mL), and then antibiotic discs were placed aseptically on it. Plates were incubated at 37°C for 24 h. After 24 h, the inhibition zones around the discs were measured. These zones were compared with standard zones according to Clinical and Laboratory Standards Institute ^[23] to classify *S. aureus* isolates as resistant, intermediate, and sensitive to various antibiotics ^[27-29].

Well Diffusion Assay for Non-Antibiotics and Resistant Antibiotics

The well diffusion assay was used for the evaluation of the effectiveness of highly resistant antibiotics and NSAIDs

alone as well as in combinations against MRSA isolates. For this purpose, bacterial culture (1×10^8 CFU/mL of activated staphylococcal growth) was evenly spread across the surface of pre-seeded MHA plates, accompanied by the well formation of 6–8 mm with the help of a sterile cork-borer^[30]. Antibiotics (Sigma Aldrich®) and NSAIDs (Nexgen®) powders were made in dilutions of specific concentrations according to guidelines of^[31] and poured alone and in combination, into wells according to desired volumes, accompanied by incubation at 37°C for 24 h following the guidelines of Ahmed et al.^[6]. The well having no dilution of drug was considered a negative control. Antimicrobial drugs diffuse in agar, thus inhibiting the growth of the bacterial strain. The zones of growth inhibition of antimicrobials were assessed according to the guidelines of Aqib et al.^[21]. After this, modulation factors were calculated using the formula^[31]. The increase in ZOI in combination, in comparison with zones of drugs alone, was deemed as effective^[32].

Data Analysis

The Thrushfield formula was used to assess the prevalence of *S. aureus* and MRSA^[33]. To analyze the association of different risk factors with staphylococcal respiratory infections, the Chi-square test and Logistic regression analysis were used at a 5% probability using SPSS version 22^[34]. The combinations of resistant antibiotics and NSAIDs were evaluated based on the modulation factor, assessed as ZOI of antibiotics divided by ZOI of the antibiotics and NSAIDs combination, as calculated by Ahmed et al.^[6]. The modulation factor <0.5 indicates synergistic interaction of resistant antibiotics and NSAIDs in inhibiting the bacterial growth.

RESULTS

Prevalence of *S. aureus* and MRSA

As per the current study, the molecular prevalence of *S. aureus* was 37.86% in cats with a history of respiratory disease. The findings indicated that *S. aureus* prevalence was higher (44.78%) in samples taken from catteries than from client-owned cats (25%). The Cefoxitin disk diffusion test confirmed a 24.27% (25/103) prevalence of MRSA phenotypically. In contrast to that, genotypic confirmation by the PCR targeting the *mecA* gene revealed that MRSA is 14.5% (15/103) prevalent (Table 1).

Analysis of Risk Factors Associated with *S. aureus* Infection in Cats

The analysis of possible risk factors using the Chi-square test revealed that respiratory illness, age, season, housing hygiene, housing type, human contact, availability of diagnostic facilities, and presence of other livestock species show a P-value <0.05 and were regarded to have a significant association with *S. aureus* infection in cats. The comparative risk factors analysis for *S. aureus* and MRSA shows that breed is insignificantly associated with *S. aureus* as well as MRSA, even though some of the risk factors have an insignificant association with *S. aureus* but are significantly associated with MRSA and vice versa. For instance, age, season, housing type, and presence of other livestock species were significantly associated with *S. aureus* infection in cats, having an insignificant association with MRSA. In contrast to that, antibiotic usage and outdoor access were significant risk factors for MRSA infection, while these were found to have an insignificant association for *S. aureus*. However, most of the risk factors were significantly associated with both *S. aureus* and MRSA, such as housing hygiene, respiratory illness, human contact, and availability of diagnostic facilities. The significant factors were considered for in-depth analysis via the Logistic Regression model (Table 2).

Regression Analysis of Significant Risk Factors

Regression analysis of significant risk factors with *S. aureus*: Significant risk factors for *S. aureus* and MRSA were analyzed in comparison. The analysis revealed that respiratory illness in cats contributes 2.72 times more towards *S. aureus* infection in the respiratory tract of cats in comparison to healthy cats. Likewise, cats reared in catteries were 2.43 times more prone to contract *S. aureus* in comparison to client-owned cats. Poor housing hygiene contributed 3.01 times more towards *S. aureus* infection in cats in comparison to good hygienic management. Cats with human contact turned out to be 2.55 times higher likelihood of *S. aureus* infection as a secondary pathogen during respiratory tract infection than cats without human contact. Similarly, there was 2.17 times more risk of *S. aureus* infection in cats that were reared along with other animal species, such as dogs, in comparison to cats that were reared alone. Cats in the winter season had 2.33 times more chances of

Table 1. Phenotypic and genotypic prevalence of *S. aureus* and MRSA in respiratory tract infections of cats

Source of Sample	No. of Samples	S. aureus Prevalence (%)		MRSA Prevalence (%)	
		Phenotypic	Genotypic	Phenotypic	Genotypic
Cattery cats	67	41	30 (44.78)	17 (25.37)	9 (13.43)
Client-owned cats	36	15	9 (25)	8 (22.22)	6 (16.66)
Total	103	56 (54.37)	39 (37.86)	25 (24.27)	15 (14.5)

Table 2. Analysis of risk factors associated with MRSA infection in cats

Variable	Variable Name	<i>S. aureus</i>			MRSA		
		Total	Positive (%)	P-value	Total	Positive	P-value
Breed	Domestic short hair	51	19 (37.25)	0.454	51	7 (13.72)	0.744
	Persian	24	10 (41.66)		24	4 (16.66)	
	Mix breed	20	9 (45.00)		20	4 (20.00)	
	Siamese	5	0 (0.00)		5	0 (0.00)	
	Himalayan	3	1 (33.33)		3	0 (0.00)	
Sex	Male	61	24 (39.34)	0.709	61	8 (13.11)	0.616
	Female	42	15 (35.71)		42	7 (16.66)	
Age	<1 year	40	23 (57.5)	0.001*	40	9 (22.5)	0.069
	>1 year	63	16 (25.39)		63	6 (9.52)	
Stocking density	Low	46	15 (32.60)	0.323	46	5 (10.87)	0.340
	High	57	24 (42.10)		57	10 (17.54)	
Season	Winter	50	24 (48.00)	0.039*	50	9 (18.00)	0.337
	Summer	53	15 (28.30)		53	6 (11.32)	
Housing Hygiene	Poor	67	31 (46.26)	0.016*	53	12 (22.64)	0.017*
	Good	36	8 (22.22)		50	3 (6.00)	
Respiratory illness	Yes	62	29 (46.77)	0.022*	62	13 (20.96)	0.023*
	No	41	10 (24.39)		41	2 (4.87)	
Outdoor access	Yes	46	20 (43.47)	0.291	43	10 (23.25)	0.034*
	No	57	19 (33.33)		60	5 (8.33)	
Housing type	Cattery	67	30 (44.78)	0.048*	67	13 (19.40)	0.057
	Client owned	36	9 (25.00)		36	2 (5.55)	
Human contact	Yes	57	27 (47.36)	0.027*	41	10 (24.39)	0.021*
	No	46	12 (26.08)		62	5 (8.06)	
Diagnostic facilities	Yes	50	25 (50.00)	0.014*	35	9 (25.71)	0.021*
	No	53	14 (26.41)		68	6 (8.82)	
Other animal spp. present	Absent	54	14 (25.93)	0.009*	43	5 (11.62)	0.475
	Present	49	25 (51.02)		60	10 (16.66)	
Antibiotic usage	Yes	54	21 (38.88)	0.822	32	8 (25.00)	0.044*
	No	49	18 (36.73)		71	7 (9.85)	

* Statistically significant risk factor

S. aureus infection than during the summer season. Also, occurrence of *S. aureus* was 2.78 times more in areas where advanced diagnostic facilities were available as compared to the areas where diagnostic facilities were lacking. There was 3.97 times more risk of *S. aureus* infection in younger cats (<1 year) as compared to older (>1 year) ones (Table 3).

Regression analysis of significant risk factors with MRSA: The results revealed that poor housing hygiene in cats accounted for 4.58 times more risk for the MRSA-associated respiratory infections than proper hygiene

management. Likewise, cats with respiratory illness were 5.173 times more at risk of MRSA infection than healthy cats. Outdoor access contributed 3.01 times more towards MRSA infection in cats in comparison to indoor cats. There was 3.67 times more risk of MRSA-associated respiratory infections in cats with frequent human contact than without human contact. Also, the occurrence of *S. aureus* was 3.57 times more in areas where advanced diagnostic facilities were available as compared to the areas where diagnostic facilities were lacking. Antibiotic usage in the past accounted for a 3.04 times higher risk for MRSA infection in cats (Table 4).

Table 3. Risk factors included in the final logistic regression model for *S. aureus* infection in cats

Variable	Response	OR	95% C.I.	S.E.	P-value
Age	<1 year	3.974	1.706, 9.256	0.4321	0.001
	>1 year	Ref			
Season	Winter	2.338	1.035, 5.285	0.416	0.041
	Summer	Ref			
Housing Hygiene	Poor	3.014	1.200, 7.569	0.470	0.019
	Good	Ref			
Respiratory illness	Yes	2.724	1.141, 6.503	0.444	0.024
	No	Ref			
Housing type	Cattery	2.432	0.994, 5.953	0.457	0.052
	Client owned	Ref			
Human contact	Yes	2.550	1.102, 5.899	0.428	0.029
	No	Ref			
Diagnostic facilities	Available	2.786	1.221, 6.355	0.421	0.015
	Not available	Ref			
Other animal spp. presence	Present	2.976	1.301, 6.806	0.422	0.010
	Absent	Ref			

OR = Odds Ratio, C.I.= Confidence Interval, S.E.= Standard Error, Ref = Reference

Phylogenetic Analysis of Sequenced Study Isolates

For phylogenetic analysis of study isolates, five representative sequences (Accession number: PP768062, PP852380, PP848319, PP848320, and PP848321) were selected from sequenced isolates based on highest similarity index and query cover when they were run on nucleotide BLAST of NCBI. These selected study sequences were matched against already documented sequences of the *Staphylococcal mecA* gene from different countries on GenBank of NCBI. Four out of five study isolates Accession

number: PP768062, PP852380, PP848319, PP848320 depicted highest similarity with each other as well as with already documented sequences of *mecA* from Myanmar (Accession number: LC727174) and Pakistan (Accession number: ON643114), moderate similarity with *mecA* sequences reported from Kenya, South Korea, Germany, Egypt, and India (accession numbers: CP141508, CP085690, CP127021, OP651956 and MH221030) while least similarity with sequences reported from Nigeria, India, Saudia Arabia, Pakistan, and Iraq (Accession

Table 4. Risk factors included in the final logistic regression model for MRSA infection

Variable	Response	OR	95% C.I.	S.E.	P-value
Housing Hygiene	Poor	4.585	1.209,17.385	0.680	0.025
	Good	Ref			
Respiratory illness	Yes	5.173	1.101,24.301	0.789	0.037
	No	Ref			
Outdoor access	Yes	3.333	1.048,10.601	0.590	0.041
	No	Ref			
Human contact	Yes	3.677	1.154,11.721	0.591	0.028
	No	Ref			
Diagnostic facilities	Available	3.577	1.156,11.072	0.577	0.027
	Unavailable	Ref			
Antibiotic use age	Yes	3.048	0.997,9.318	0.570	0.051
	No	Ref			

P-value <0.05=significant results

OR = Odds Ratio, C.I.= Confidence Interval, S.E.= Standard Error

number: OK040766, MK125082, KY467026, MW558050, KX870024, and MZ359763). However, the remaining one study isolate (Accession number: PP848321) has shown significant variation from remaining study isolates but it has shown highest similarity with *mecA* sequences from Nigeria, India, Saudia Arabia, Pakistan, and Iraq (Accession number: OK040766, MK125082, KY467026, MW558050, KX870024, and MZ359763) (Fig. 4).

Antibiogram Profiling of *S. aureus* Isolates

Antibiotic susceptibility testing revealed that (100%) study isolates showed resistance against three antibiotics, including Penicillin, Cefixime, and Ceftriaxone while 75% of study isolates showed sensitivity against oxytetracycline and doxycycline. Study isolates showed the highest sensitivity (91.6%) against Trimethoprim/Sulphamethoxazole and gentamicin. Isolates showed considerable resistance against other antibiotics such as Ampicillin (75%), Vancomycin (75%), Cephalexin (66.7%), Amoxicillin (41.6%), Tylosin (33.3%), and Ciprofloxacin (25%) (Table 5).

Well Diffusion Assay for Non-Antibiotics and Resistant Antibiotics

Combination of resistant antibiotics (Ceftriaxone, Cefixime, and Penicillin) with minimum inhibitory concentrations (MIC) of NSAIDs showed a considerable increase in the zones of inhibition, as previously reported by Mansoor et al.^[35]. In case of Ceftriaxone, Ketoprofen

exhibited synergistic activity with Ceftriaxone, having modulation factor of 0.48. Ketoprofen also showed synergism with Cefixime as well (modulation factor = 0.44). Meloxicam also resulted in a considerable increase in ZOI when combined with Penicillin G, with a modulation factor of 0.48 (synergistic effect). The combination of the remaining two antibiotics (Ceftriaxone and Cefixime) with meloxicam and the combination of Penicillin G with Ketoprofen did not show synergism (Table 6).

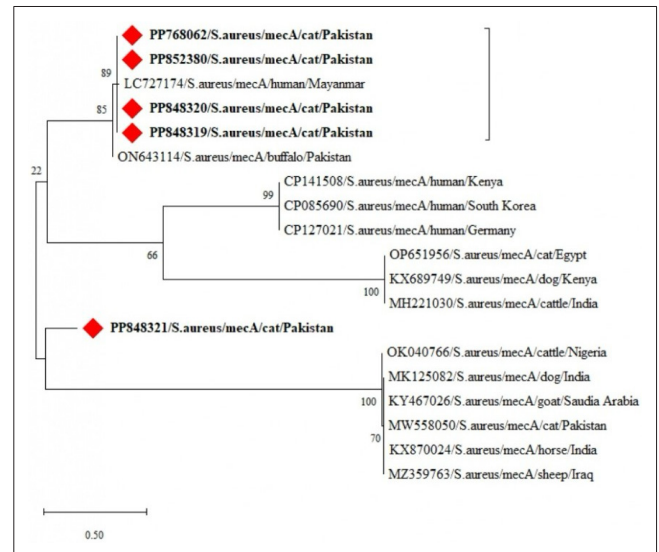


Fig 4. Phylogenetic analysis of staphylococcal *mecA* gene isolated from cats. The isolates with red bullets indicate study isolates

Table 5. Antimicrobial susceptibility profiling of MRSA isolates

Antibiotics	Abbreviations	Breakpoint Zone Diameter (mm)			<i>mecA</i> Positive Isolates (n=12)		
		R	I	S	R (%)	I (%)	S (%)
Penicillin G	P	≤28	-	≥29	12 (100)	0 (0)	0 (0)
Amoxicillin	AMX	≤16	-	≥23	5 (41.6)	5 (41.6)	2 (16.66)
Ampicillin	AM	≤28	-	≥29	9 (75)	3 (25)	0 (0)
Cephalexin	CL	≤14	15-17	≥18	8 (66.7)	2 (16.66)	2 (16.66)
Ceftriaxone	CRO	≤13	14-20	≥21	12 (100)	0 (0)	0 (0)
Cefixime	CFM	≤15	16-18	≥19	12 (100)	0 (0)	0 (0)
Gentamicin	CN	≤12	13-14	≥15	1 (8.33)	0 (0)	11 (91.6)
Tylosin	TY	≤14	15-18	≥19	4 (33.33)	3 (25)	5 (41.6)
Oxytetracycline	T	≤14	15-18	≥19	1 (8.33)	2 (16.66)	9 (75)
Doxycycline	DOX	≤12	13-15	≥16	2 (16.66)	1 (8.33)	9 (75)
Ciprofloxacin	CIP	≤15	16-20	≥21	3 (25)	6 (50)	3 (25)
Levofloxacin	LEV	≤15	16-18	≥19	1 (08.33)	2 (16.66)	9 (75)
Trimethoprim + Sulphamethoxazole	TMS	≤10	11-15	≥16	1 (08.33)	0 (0)	11 (91.6)
Vancomycin	VA	≤17	18-20	≥21	9 (75.00)	3 (25)	0 (0)

R: Resistant; I: Intermediate; S: Sensitive

Table 6. Results of antibiotic resistance modulation by NSAIDs using the well diffusion assay

Resistant Antibiotics	NSAIDs	ZOI for Antibiotics Alone (mm)	ZOI for NSAIDs Alone (mm)	Zone of Inhibition in Combination (mm)	Modulation Factor
Ceftriaxone	Ketoprofen	12	17	25	12/25=0.48
	Meloxicam	13	10	17	13/17=0.76
Cefixime	Ketoprofen	8	14	18	8/18=0.44
	Meloxicam	10	9	12	10/12=0.83
Penicillin	Ketoprofen	22	18	29	22/29=0.76
	Meloxicam	15	20	31	15/31=0.48

*ZOI = Zones of inhibition; Modulation Factor <0.5=Synergism

DISCUSSION

S. aureus is a commensal bacterium in animals as well as humans since it usually inhabits the respiratory tract, especially nares, in healthy animals; however, it also causes diseases that may be even fatal [36]. Cats live near human beings and can transmit pathogens of zoonotic importance [37]. This study was done to assess the prevalence of methicillin-resistant *S. aureus* both phenotypically (Disc diffusion method) and genotypically (PCR targeting the *mecA* gene) in the upper respiratory tract of cats. This study is conducted for molecular characterization, especially focusing on sequencing and phylogenetic analysis of MRSA residing within the respiratory tract of cats in Pakistan.

In this study, *S. aureus* was found to be 37.86% prevalent on the molecular basis in cats. It is about double that reported by Bierowiec et al. [11]. This higher prevalence may be due to the adoption of poor housing hygiene practices, especially in cats reared in catteries. *S. aureus* was found to be more prevalent in catteries than client-owned cats. This is possibly due to higher stocking density, poor ventilation, and hygiene conditions in catteries. Previously a research conducted by Habibullah et al. [38] in Dhaka reported a prevalence of 40.86% in pets, including dogs and cats. The prevalence reported by Lilenbaum et al. [39] in cats of Brazil is about one-fourth (9.4%) of that of the current study. A research done by Bierowiec et al. [40] to evaluate comparative prevalence of different *Staphylococcal* species from different anatomical sites in cats revealed a prevalence of 7.37% for *S. aureus* in cat nares. In a comparative study by Moon et al. [41] to evaluate the burden of different pathogens in respiratory samples of cats, *S. aureus* was not detected as a significantly prevalent species.

In the present study, methicillin-resistant *Staphylococcus aureus* (MRSA) was phenotypically detected in 24.27% of isolates using the Cefoxitin disc diffusion method. Previously, phenotypic prevalence of MRSA was reported to be 26.6% in Philadelphia [42], which is comparable to the results of the current study. MRSA prevalence carriage was

reported to be 0.46% and 2.16% in apparently healthy and diseased cats, respectively, in Greater London [43], which is quite less than the current study. The possible reason behind this difference may be poor housing hygiene and irrational use of antimicrobial agents in Pakistan. The MRSA prevalence reported by Habibullah et al. [38] in Dhaka aligns with the current findings, which may be attributed to the shared geographical region. Previously, Shoaib et al. [20] reported a 30.43% phenotypic prevalence of MRSA in cats in Pakistan using the Oxacillin disc diffusion method, which is marginally higher than the prevalence observed in the present study.

Genotypically MRSA was 14.5% prevalent in this study. Previously, genotypic prevalence in Pakistan was reported at 30.34% in cats based on PCR targeting the *mecA* gene [20], which is about double that of the current results. Moreover, the phenotypic and genotypic prevalence of MRSA were similar in that study, in contrast to our study results, where phenotypic (24.27%) and genotypic (14.5%) prevalence had a significant difference. This difference may be due to a discrepancy between phenotypic and genotypic methods to detect MRSA. This discrepancy indicates that Cefoxitin is not an effective alternative for identifying the *mecA* gene [13]. In a study conducted in Libya, the PCR-based confirmation for *mecA* revealed the prevalence of 13% in cats [44], which is very close to our study results. In another study conducted in Dhaka, MRSA was confirmed by PCR in 5.88% of cats [38], which is about one-third of our study results. This difference may be due to poor hygienic measures, environmental changes, excessive human contact, and irrational antimicrobial use age in Pakistan.

Antibiogram profile of current study isolates showed that isolates were highly resistant against Penicillin G, Ceftriaxone, and Cefixime. This resistance may be due to the excessive antibiotics in some countries [45] and different genetic mutations that the microorganisms have acquired over time [46]. This study showed that the susceptibility results slightly contradict the findings of Shoaib et al. [20]. In the current study, 25% of isolates were resistant to ciprofloxacin, in contrast to the Shoaib et al. [20] where all

the isolates (100%) were sensitive to ciprofloxacin. Resistance against vancomycin was also higher (75%) in comparison to the results of Shoaib et al.^[20]. Susceptibility findings of the current study isolates were similar to results reported by Anwaar et al.^[17] in case of Tylosin, while sensitivity against oxytetracycline was higher (75%) in the current study. When the study results were compared to those reported by Rasheed et al.^[14], it revealed that results vary significantly in case of Trimethoprim/Sulphamethoxazole while results are almost similar in case of Amoxicillin.

Phylogenetic analysis of the *mecA* revealed substantial similarity within study isolates and the isolates from various other countries (Myanmar, India, Saudi Arabia, Iraq, Nigeria, Egypt, Germany, South Korea, and Kenya). The analogy between local isolates and isolates from different countries indicated that there is dissemination of MRSA between countries, possibly due to trade of animals, especially import of pet animals, and transboundary mobility of human beings, leading to worldwide presence of these notorious pathogens^[17].

Resistance modulation trials using different combinations of NSAIDs and antibiotics showed synergistic effects. In a study, Bakht et al.^[47] found that NSAIDs increased the zones of inhibition of resistant antibiotics when antibiotics were administered in combination with various NSAIDs to combat MRSA. As some NSAIDs also have antimicrobial activities along with their antipyretic, analgesic, and anti-inflammatory activities^[48]. So, this approach can be used to resensitize MRSA against commonly used antibiotics. Synergistic activity of antibiotics in combination with NSAIDs against MRSA has also been well established by Aqib et al.^[31] Altaf et al.^[49], and El-Deeb et al.^[50], and which can be because of bacteria targeting various sites^[49].

Furthermore, this study revealed a significant association of different risk factors that is season, age, respiratory illness, housing hygiene, housing type, human contact, availability of diagnostic facilities, and presence of other animal species, with *S. aureus* infection in the respiratory tract of cats. The findings of various risk factor associations turned out to be similar to the findings as per Bierowiec et al.^[11] and Shoaib et al.^[20]. For instance, the presence of other livestock species, human contact, and availability of diagnostic facilities were significant risk factors in both of these studies and the current study as well. Some of the risk factors were very peculiar as they were reported novel in this study, such as housing hygiene and respiratory illness. The association of housing hygiene with *S. aureus* infection in cats may be due to poor hygienic measures^[51] and likewise association of respiratory illness with *S. aureus* infection may be due to immunosuppression due to viral infections that leads to

S. aureus infection as a secondary pathogen^[52]. Housing type was also found to be significantly associated with *S. aureus*. This is because of close contact of cats with each other, especially in catteries^[17]. Comparative risk factors analysis for *S. aureus* and MRSA exhibited that frequent use of antibiotics is significantly associated with MRSA, but it is an insignificant risk factor for *S. aureus* infection in cats. This significant association of antibiotic usage may be due to resistance acquired by the bacterium, which helps it to evade the action of antibiotics and helps it to colonize the respiratory tract. In contrast to that, season turned out to be a significant risk factor associated with *S. aureus*, while it is insignificant for MRSA infection. This is possibly due to better conditions for *S. aureus* to colonize during the winter season^[53].

In conclusion, this study revealed that MRSA is a significantly prevalent pathogen in cats with respiratory tract infections. However, these *S. aureus* infections can be controlled in cats by controlling various risk factors, which turned out to be associated significantly with occurrence of *S. aureus* infections according to the study, such as better management practices like appropriate hygiene, good housing, and proper treatment of respiratory illness can control MRSA infection in cats. This study demands an in-depth investigation into the transmission of MRSA from companion animals to humans and the environment from the perspective of one health. Furthermore, well diffusion assay of combinations (Resistant antibiotics + NSAIDs) revealed significant resistance modulation in MRSA. This combinational therapy (Antibiotics + NSAIDs) can be implemented as an alternative therapeutic option against antimicrobial-resistant strains of *S. aureus* in the future.

This study is subject to certain limitations. The sample collection was restricted to a limited number of veterinary clinics and geographic locations, which may not adequately represent the overall feline population in Pakistan, thereby potentially limiting the phylogenetic and antibiogram analysis. Furthermore, the molecular detection strategy relied on PCR assays targeting established resistance determinants, such as the *mecA* gene, which may preclude the identification of novel or less-characterized resistance genes and genetic variants, leading to an underestimation of the true diversity of methicillin resistance. In addition, antimicrobial susceptibility testing performed using disk diffusion may have limited sensitivity in detecting certain phenotypic expressions of resistance. It is also important to note that in vitro susceptibility profiles may not consistently translate into in vivo therapeutic efficacy, as host-related and environmental factors can significantly influence antimicrobial performance, thereby constraining the direct clinical application of the observed resistance patterns.

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RESEARCH ARTICLE

LC-MS-Based Phytochemical Analysis and Anthelmintic Potency of *Cassia fistula* (Linn.) Extracts Against Gastrointestinal Nematodes of Sheep and Goats

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Abstract

Medicinal plants play an important role in addressing global health challenges in both humans and livestock, particularly those associated with gastrointestinal parasitic infections. The present study aimed to conduct comprehensive LC-MS-based phytochemical profiling of various *Cassia fistula* extracts and their anthelmintic activity against gastrointestinal parasites in sheep and goats. *In vitro* anthelmintic efficacy of crude extracts prepared from different plant parts (leaves, stem, fruit, and root) was evaluated using two-fold serial dilutions, with 50 mg/mL as the stock concentration. Levamisole, Oxfendazole, and Albendazole were used as positive controls, and phosphate-buffered saline (PBS) as the negative control. Based on *in vitro* performance, the ethanol fruit extract and crude fruit powder were further assessed *in vivo* in goats (n=40) at dose rates of 250, 500, and 1000 mg/kg body weight. Phytochemical characterization of all extracts was carried out using LC-MS analysis under both positive and negative electrospray ionization modes. Treatment with the crude ethanol fruit extract against *Haemonchus contortus*, resulted in mortality of all adult parasites within 4 h and 90.33% inhibition of egg hatching at 50 mg/mL, which was comparable and significantly higher than the positive controls (P<0.05). In case of the *in vivo* study at Day 21, crude fruit ethanol extract with the highest dose (1000 mg/kg) significantly reduced parasite burden of 90.78±0.55%, which was comparable to ABL (87.91±2.08%) (P>0.05). A total of 220 compounds were identified in LC-MS analysis of *C. fistula* including anti-parasitic constituents; quercetin, kaempferol, gallic acid, and hydroxycinnamic acid derivatives. These findings suggest that *C. fistula* fruits possess notable anthelmintic potential as a natural alternative to synthetic anthelmintics. However, further studies, including detailed toxicity assessments and dose optimization, are required to establish their safety and therapeutic applicability in livestock.

Keywords: Anthelmintic efficacy, *C. fistula*, Gallic acid, *H. contortus*, LC-MS analysis

INTRODUCTION

Livestock growth and productivity are adversely impacted by the economic burden of gastrointestinal parasites, which lead to substantial losses through reduced growth, shortened lifespan, infertility, and decreased survival rates ^[1]. Among gastrointestinal helminth infections, haemonchosis holds major global economic significance,

as it restrains the fecundity and the sustainability in small ruminant populations ^[2]. This disease affects the host's immune function, hematological profile, and biochemical parameters, ultimately resulting in reduced digestion, constant blood loss, protein depletion, and eventually causing host death ^[3]. The primary causative agent of Haemonchosis, *Haemonchus contortus* (*H. contortus*), cause significant economic loss to livestock



across temperate and tropical zones, estimated to reach billions of dollars annually to the veterinary market [4]. It is considered one of the most resistant parasitic species due to its high pathogenicity and prolific reproductive capacity [5].

Parasite control has traditionally relied on synthetic anthelmintics, along with dietary and grazing management strategies [6]. However, synthetic anthelmintics are often associated with toxicity issues and adverse effects, including gastrointestinal disturbances, hepatotoxicity, neurotoxicity, and reduced productivity, as well as high costs and limited availability [7]. Moreover, the multi-resistant veterinary parasite, *H. contortus*, has developed resistance to nearly all widely used anthelmintic classes, including imidazothiazoles, ivermectin, benzimidazoles, and thiabendazole [8]. Therefore, Plant-derived anthelmintics are particularly promising because they tend to be less toxic, widely accessible, cost-effective, and easy to prepare and administer [9]. Nature offers a diverse range of bioactive compounds in plants, such as alkaloids, flavonoids, terpenoids, amino acids, sugars, and polyacetylenes that serve as defense mechanisms against bacteria, fungi, pests, viruses, and insects [10]. Moreover, the use of medicinal plants for managing parasitic infections has a long-standing historical association with both livestock and human health [11].

Advanced standardizing techniques are increasingly employed to identify secondary metabolites in plants and to evaluate their anthelmintic potential [12]. Liquid chromatography mass spectrometry (LC-MS) serves as a crucial analytical tool for the identification, quantification, and mass study of a wide spectrum of semi-volatile and volatile organic and inorganic compounds in a complex mixture [13]. The anthelmintic efficacy of medicinal plants, including different parts of the same plant, varies depending on their bioactive constituents, as well as factors such as geographical origin, cultivar, seasonal variations, and the solvents used for extraction [11].

Cassia (*C.*) *fistula* L. (commonly known as Amaltas or Golden shower) is an angiosperm plant belonging to the family Fabaceae. It is an average-sized ornamental tree characterized by its yellow cheerful flowers and dark green compound leaves [14]. In the Cholistan desert, local nomadic communities known as Roohelay traditionally used this tree as a medicinal and therapeutic moderator to support their livestock's health, growth, and productivity [15]. Previous findings have reported the anthelmintic activity of *C. fistula* leaves, where ethanol and n-hexane extracts demonstrated inhibitory effects against L3 infective larvae of *H. contortus* in larval migration inhibition assay [16]. *C. fistula* fruits may offer a rich reservoir of phyto-constituents possessing significant anthelmintic potential; hence, they may offer a promising natural alternative to

commercial drugs for the proper control and management of gastrointestinal parasitic diseases.

MATERIAL AND METHODS

Ethical Approval

Animals experiments were conducted in strict adherence to the the guidelines for the Welfare and Ethical Review committee of Laboratory Animals as well as Institutional Animal Care and Use Committee (IACUC). The experiments were conducted following approval from the Animal Ethics' Committee of the Islamia University of Bahawalpur (Approval # 456.ORIC). The university-appointed research reviewers evaluated all relevant ethical considerations and confirmed that the study procedures adhered to established animal welfare standards.

Plant Parts Collection and Handling

C. fistula fruits, fresh roots, and leaves with attached soft stem branches (about 2kg) were collected between January and February 2023, from the Cholistan desert area of the Islamia University of Bahawalpur, Baghdad campus, Punjab, Pakistan. Then, the plant samples were taxonomically identified and authenticated as *C. fistula* (voucher no. 18_01_025) at the botany department of agriculture university, Faisalabad, Pakistan. The collected plant samples were separated and thoroughly washed with fresh water to remove dust and contaminants. They were then shade-dried at a room temperature of 25°C, chopped, and ground into coarse particles using a mechanical grinder.

Extract Preparation of *C. fistula*

The plant collected samples were sequentially extracted in ethanol, methanol, aqueous methanol, and ethyl acetate following slight adjustments [17,18]. The selection of these solvents was based on previously reported literature demonstrating that solvent polarity plays a critical role in the efficient extraction of diverse phytochemical classes [19]. In particular, polar solvents such as methanol and aqueous methanol are effective for extracting polar compounds (e.g., flavonoids, glycosides), while ethanol and ethyl acetate facilitate the extraction of moderately polar to semi-polar bioactive constituents, thereby ensuring a broad phytochemical profile relevant to biological activity [19].

Approximately 100 g of powdered material from each plant part was soaked in 400 mL of ethanol in a sealed container and intermittently stirred over three days. Then, filtration of the mixture was done, and the remaining plant residue was re-soaked in another 400 mL of ethanol. This process was repeated two additional times. Then, all filtrates were mixed, and the solvent was evaporated under reduced pressure at 40°C in a rotary evaporator. The resulting crude ethanolic extract, obtained in syrup form, was stored at 4°C until further study. The same procedure was

reiterated to prepare extracts in ethyl acetate, methanol, and 75% aqueous methanol (methanol-water ratio, 3:1).

Liquid Chromatography and Mass Spectrometry (LC-MS) Study

LC-MS analysis was performed following the method described by Kaur et al.^[20]. All analyses were carried out using advanced instrumentation at International Laboratories of Textile Testing (TTI), Lahore, Pakistan. The chromatographic method employed was a water eluent in UPLC H-Class, prepared with a BEH-C18 column (2.1 × 100 mm, 1.7 µm particle size). This UPLC platform combines the sensitivity, high speed, and resolution of UPLC with the operational flexibility of conventional HPLC. The mobile phases (each of 0.1%) consisted of formic acid in water (mobile phase A) and formic acid in acetonitrile (mobile phase B) with a flow rate of 0.25 mL per minute. A gradient elution profile was applied to optimize compound separation, with the following solvent ratios (Time - A/B): 0 min -90/10, 2 min -90/10, 5 min -80/20, 10 min -70/30, 12 min -50/50, 14 min -90/10. A sample volume of 4 µL was injected into the column, and the column temperature was maintained at 30°C. The injection volume was kept constant across all analyses to ensure methodological consistency. This low volume of sample was used to prevent peak broadening and to maintain high resolution, which is critical for narrow-bore UPLC columns.

Mass spectrometric detection was conducted in a TOF-MS (time of flight mass) system equipped with an electrospray ionization (ESI) source. Instrument parameters included: cone gas and desolvation gas flows of 30 and 850 L/h (nitrogen/argon), respectively, desolvation and source temperature (450 and 120°C), capillary and cone voltage (3.21 kV and 50V), respectively, and collision energy of 4eV. Each extract was analyzed in both negative (-) and positive (+) ionization modes. All LC-MS analyses were performed using a Waters UPLC-QTOF MS system (Waters Corporation, Milford, MA, USA), ensuring methodological reproducibility. Plants' phytochemicals identification was based on LC retention time and accurate mass measurements. When available, reference standards were used for compound identification by matching both exact mass and retention time. However, the tentative identifications were made using accurate mass data, published literature, and characteristic fragmentation patterns of well-known metabolite classes.

Anthelmintic Assessment of Plant Extracts and Positive Control Groups

Adult Worms' Motility Inhibition Assay

The basic methodology of adults' motility inhibition assay was carried out following the method with slight

adjustments^[17,18]. The *in vitro* assays were conducted using a well-characterized and biologically relevant model parasite, *H. contortus*. This parasite species was specifically selected because it is recognized as one of the most prevalent and highly pathogenic gastrointestinal nematodes affecting small ruminants worldwide, including in our region. The active, energetic, and mature worms of *H. contortus* were collected instantly after evisceration from the abomasum of a naturally infected, freshly slaughtered sheep from a local slaughterhouse and transferred into phosphate-buffered saline solution (PBS). Actively motile adult worms (n=10) were placed into separate Petri dishes containing various plant extract concentrations (50, 25, 12.5, 6.25, 3.125 mg/mL), along with a negative control treatment (PBS; 20 mL per dish) and a positive control assay (Levamisole; 0.55 mg/mL). Levamisole was used in the adult motility inhibition assay because it is a well-established reference drug for evaluating neuromuscular paralysis in adult nematodes. The different doses of various plant extracts were prepared through two-fold serial dilutions in PBS, using 50 mg/mL as the stock concentration.

All assays were executed in triplicate and repeated three times at 25°C. The worm's motility was evaluated under an inverted microscope following 2 h intervals for a duration of 12 h to determine the anthelmintic activity of *C. fistula* extracts. Worms exhibiting no movement at the head or tail, were transferred to lukewarm saline for approximately 5 min to confirm their motility inhibition. The percentage worm's motility was measured by following the formula described by Resendiz-Gonzalez et al.^[21].

Egg Hatch Assays

Egg hatch inhibition assays were conducted following the process of "World Association for the Advancement of Veterinary Parasitology (WAAVP)," with minor adjustments^[17]. The mature female worms of *H. contortus* were recognized based on their larger size (18-30 mm) and characteristic reddish appearance due to the white uterus spiraling around the red gut, giving the typical barber-pole pattern. Fully mature females were washed with PBS solution and then homogenized in a mortar containing 5 mL of PBS to release the eggs. The resulting suspension was filtered through an 80-µm mesh sieve under gentle gravity (without applied pressure) to remove tissue debris, and no fixed duration was required for this step. The filtrate was then diluted with PBS to obtain an egg suspension of approximately 200 eggs/mL.

All the treatments were tested in triplicate and repeated thrice in 24 multi-well plates using two-fold serial dilution starting from 50 mg/mL stock solution (50, 25, 12.5, 6.25, and 3.125 mg/mL) for plant extracts and 50 µg/mL stock solution for positive control, Oxfendazole. A negative

control, PBS were included in the assay. Oxfendazole was selected for the egg hatch assay due to its strong ovicidal activity and its frequent use as a standard control in egg hatch inhibition studies. Oxfendazole concentrations were taken in µg/mL, and plant extract doses in mg/mL. The eggs' incubation in labeled plates was carried out at 70% relative humidity and 27°C for 48 h. After incubation, unhatched eggs and hatched larvae of either live or dead were recorded under a compound microscope using 40× magnification power. The percentage egg hatch inhibition rate was determined by applying the formula described by Dhruw et al.^[22].

In Vivo Bioassays

In vivo anthelmintic evaluation was conducted using only the *C. fistula* fruit powder and ethanol fruit extract, as the fruit ethanol extract demonstrated the highest efficacy during the *in vitro* screening. For extract preparation, 1 kg of fruit powder was immersed in 3 L of ethanol, intermittently stirred, and then filtered through a spongy cloth. The same procedure was reiterated thrice, the filtrates were mixed, and then concentrated in a rotary evaporator. The resulting syrup form extract was stored at 4°C. The *in vivo* assessment was subsequently carried out using the method followed by Flores-Prado et al.^[23].

Experimental Design

For the *in vivo* study, goats (n=40) of either sex, male and female, and naturally infected with mixed gastrointestinal parasitic species for over six months, were selected randomly. Consequently, the experimental animals were not artificially infected with a single parasite species, but instead represented a realistic field scenario where multiple nematode species coexist and contribute collectively to the parasite burden. All the animals had a weight between 16 and 25 kg and were aged 7-10 months. Throughout the study, they were maintained on an adequate supply of food, fodder, and concentrates. The goats were randomly assigned to eight groups of five animals each. Group 0 (T₀) served as the untreated control. Group 1 (T₁) was treated with a single oral dose of albendazole (10 mg/kg b.wt). Albendazole was chosen as the positive control because it is widely used in field conditions for the treatment of gastrointestinal nematodes in small ruminants and thus served as a practical therapeutic benchmark. Ethanol fruit extract was administered to groups 2, 3, and 4 (T₂, T₃ & T₄) correspondingly at dosage rates of 250, 500, and 1000 mg/kg b.wt. While the groups 5, 6, and 7 (T₅, T₆, T₇) were treated with fruit powder at the same corresponding dose levels (250, 500 & 1000 mg/kg b.wt.). All the enrolled goats exhibited substantial gastrointestinal nematode burden, as verified using the McMaster fecal eggs calculation method.

Fecal samples from each goat's rectum were collected

usually in the morning, starting from the day before treatment (D₀) to post treatment days (D₇, D₁₄ & D₂₁). For egg count assessment, approximately 2 g of feces were mixed in 30 mL of tap water and homogenized, then mixed with 30 mL of PBS solution. A small portion of this mixture was transferred to a McMaster counting chamber using a pipette and studied under a microscope at 10× magnification to evaluate the number of nematode eggs. Accordingly, Belga et al.^[24] calculated the parasite egg reduction.

Statistical Analysis

The data recorded in both bioassays, *in vitro* and *in vivo* was analyzed using one-way ANOVA in SPSS. Significant differences among treated and untreated groups were determined using the Duncan multiple range test.

RESULTS

Percentage of the Extracts' Yield

The solvent extraction of various extracts of *C. fistula* produced the following yields: fruit's ethanol, methanol, ethyl acetate, aqueous-methanol extracts (12.5, 13, 9.2, 8%), roots ethanol, methanol, aqueous-methanol, ethyl acetate extracts (6.5, 9, 7.4, 8.5%), stem ethanol, methanol, ethyl acetate, aqueous-methanol extracts (9.8, 7, 9.1, 7.5%), leaves ethanol, methanol, ethyl acetate, aqueous-methanol extracts (11, 11.5, 7.5, 7.5 %) respectively. Among all extracts, the highest yield was obtained from the fruit's methanol extract, whereas the lowest yield was observed for the root ethanol extract. In case of the *in vivo* study, the ethanol fruit extract produced a yield of 14.25%. The extract percentage yield was calculated as the dried extracts' weight divided by the initial plant powders' weight, multiplied by 100 (% w/w *100).

Liquid Chromatography and Mass Spectrometry (LC-MS) Study of *C. fistula*

LC-MS profiling of extracts from different parts of *C. fistula* identified numerous major classes of pharmaceutical compounds, including alkaloids, glycosides, terpenoids, phenols, and flavonoids. The active pharmaceutical constituents detected in only trace amounts in the leaves extracts were excluded from [Table 1](#). Adicardin, myricetin, adenosine diphosphate, phytol sodium, lauric acid, pyroglutamic acid were present in trace amounts in leaves ethanol extract; cycloartenol acetate, catalpol sodium, succinylproline, 3-indoleacrylic acid in leaves methanol extract; myricetin, arachidonic acid, cycloartenol acetate, vomifoliol, dihydroquercetin in leaves ethyl acetate extract; quercetin-3'-O-glucoside, coniine, levulinic acid in aqueous-methanol extract. Similarly, compounds present in minimal quantities in the stem extracts were not included in [Table 2](#). These included campesterol, propyl gallate,

Table 1. Phytochemical compounds detected in LC-MS study in different <i>C. fistula</i> leaves' extracts						
Phyto-Constituents		Class Name	R.T. (min.)	% Area	M.W. (g/mol)	% Abund.
Bioactive metabolites present in leaves' ethanol extract	Quercetin	Flavonoids	2.75	1.89	302.2	3.33
	Proanthocyanidin B-dimers'	Flavonoids	5.31	4.14	578.5	1.14
	Vicenin-2	Flavonoids	0.69	2.13	594.5	2.11
	Gallic Acid	Phenolic Acids	1.59	1.23	170.12	1.06
	Apigenin-6C-Pentoside-8C-hexoside (isomer I)	Flavonoids	1.81	1.54	564.5	5.30
	Syringaresinol	Lignan	1.52	5.31	418.4	2.97
	Coumaric Acid	Phenolic Acids	2.91	5.75	308.5	1.67
	Ferulic Acid	Phenolic Acids	6.77	7.93	194.2	3.04
	Myricetin Hexoside	Flavonoids	1.79	7.46	480.4	3.33
	Kaempferol Rhamnosyl Xyloside	Flavonoids	3.34	4.24	564.5	1.73
	3,4-Di-O-Caffeoylquinic Acid	Phenolic Acids	3.48	2.68	516.5	1.06
	Astragalin	Flavonoids	1.11	1.21	448.4	1.96
	Protocatechuic Acid-4O-glucoside	Phenolic Acid	0.82	1.31	316.3	1.73
Bioactive metabolites present in leaves' methanol extract	Kaempferol 3O-Acetyl-Glycoside	Flavonoids	6.21	1.21	490.4	1.96
	p-Coumaric Acid	Phenolic Acids	3.49	2.56	164	2.90
	Vitexin	Flavonoids	1.52	3.44	432..4	1.60
	Rutin	Flavonoids	1.52	6.40	610.5	1.25
	Alpha-Linolenic Acid	Fatty Acids	2.31	3.53	278.4	4.04
	Apigenin-6C-Pentoside-8C-hexoside (isomer II)	Flavonoids	0.81	5.91	564.5	2.84
	Quercetin-3O-Hexoside	Flavonoids	7.71	6.05	463.4	2.99
	Neochlorogenic Acid	Polyphenols	5.29	4.88	354.3	1.46
	Quercetin 3O-2Acetyl-Glucoside	Flavonoids	2.91	6.71	506.4	7.19
	Syringaresinol	Lignan	6.57	6.64	418.4	3.91
	Apigenin-6C-Pentoside-8C-hexoside (isomer III)	Flavonoids	2.67	9.39	564.5	2.68
	Isorhamnetin-3O-Glucoside	Polyphenols	6.01	5.53	478.4	1.86
	Isorhamnetin	Flavonoids	8.37	7.42	316.2	6.27
Bioactive metabolites present in leaves' ethyl acetate extract	Vicenin 2	Flavonoids	2.25	7.47	564.5	4.04
	Trigonelline	Alkaloids	5.53	8.17	137.1	2.14
	Kaempferol Rhamnosyl Xyloside	Flavonoids	4.80	3.34	564.5	3.07
	L-Valine	α -Amino Acids	6.04	4.14	117.1	4.54
	Apigenin-6C-pentoside-8-Chexoside (isomer II)	Flavonoids	6.47	1.36	564.5	4.52
	p-Coumaric Acid	Phenolic Acids	3.81	2.31	308.5	2.10
	3,4-Di-O-Caffeoylquinic Acid	Phenolic Acids	1.91	1.67	516.5	6.21
	Myricetin Hexoside	Flavonoids	1.09	1.73	480.4	3.65
	Proanthocyanidin B-dimers'	Flavonoids	2.48	1.14	578.6	2.63
	Quercetin 3O-Hexoside	Flavonoids	4.71	3.05	463.4	5.00

Table 1. Continue

Phyto-Constituents		Class Name	R.T. (min.)	% Area	M.W. (g/mol)	% Abund.
Bioactive metabolites present in leaves' aqueous methanol extract	Apigenin-6,8-Di-C- Glycoside	Flavonoids	5.51	3.06	564.5	3.11
	Apigenin-6C-Pentoside-8-Chexoside (1 st isomer)	Flavonoids	1.75	1.26	564.5	4.48
	Apigenin-C-Hexoside-O-Pentoside	Flavonoids	2.05	1.48	270.2	1.88
	Apigenin-6C-pentoside-8C-hexoside (2 nd isomer)		1.24	2.33	564.5	1.65
	Trigonelline	Alkaloids	1.70	1.61	137.1	2.67
	Vicenin-2	Glycosides	2.11	1.22	594.5	8.53
	3,4-Di-O-Caffeoylquinic Acid	Phenolic Acids	1.92	1.40	516.5	2.68
	Isoquercitrin	Flavonoids	1.74	4.11	464.4	4.90
	Kaempferol-3O-Rutinoside	Glycosides	1.61	1.83	594.6	2.02
	Quercetin 3O-Hexoside	Flavonoids	2.61	1.24	463.4	1.44
	Kaempferol	Flavonoids	1.78	1.61	286.2	2.30

RT: Retention time, MW: Molecular weight, abund.: abundance

prenylated chalcone in ethanol extract; myrin succinyl, myricetin-3-O-xyloside, vanillic acid 4-sulfate, rutin acetate in methanol extract; asparagine, leucyl alanine, phytol in ethyl acetate extract; caffeine, pterostilbene, pallidol malonyl in aqueous-methanol extract.

A total of 14 phyto-constituents were identified in the root ethanol extract (1 phenol, 2 flavonoids, 3 phenolic acids, 4 terpenoids, 1 amino acid, 2 fatty acids, 1 stilbenoid), 13 in root methanol extract (1 amino acid, 1 steroid, 1 alkaloid, 1 glycoside, 4 flavonoids, 1 phenolic acid, 2 terpenoids, 2 polyphenols), 14 in root ethyl acetate extract (1 alkaloid, 2 flavonoids, 1 amino acid, 2 fatty acids, 4 phenols, 3 terpenoids), 14 in aqueous methanol extract (1 alkaloid, 3 flavonoids, 3 phenols, 3 fatty acids, 1 terpenoid) as shown Table 3. The fruit extracts of *C. fistula* contained several key classes of bioactive compounds, including phenols, flavonoids, alkaloids, terpenoids, and glycosides. Additionally, other classes of compounds, such as amino acids, fatty acids, and lignans, also partly contributed to the medicinal value of fruit extracts of *C. fistula* (Table 4).

Effects of *C. fistula* Extracts Against the Adults of *H. contortus*

The adulticidal evaluation of various *C. fistula* extracts, compared with Levamisole and PBS, was demonstrated in adult motility assay (Fig. 1, Fig. 2, Fig 3, Fig. 4). Fig. 1 and Fig. 2 illustrate the time-dependent paralysis and mortality of *H. contortus* exposed to different concentrations of fruit and root extracts of *C. fistula*, respectively. While Figures 3 and 4 are presenting the corresponding effects of stem and leaves extracts of *C. fistula* in comparison with Levamisole and PBS controls. At the highest concentration (50 mg/mL), the fruit ethanol extract and leaves ethyl acetate extract demonstrated the most pronounced effects, causing

complete paralysis and mortality of adult *H. contortus* (10.00 ± 0.00) within 4 and 6 h, respectively. Levamisole caused complete paralysis of all adult worms (10.00 ± 0.00) within 2-4 h of exposure, whereas no immotile worms ($0.00 \pm 0.00a$) were observed in PBS during the 12-h assay. These effects were statistically comparable to Levamisole ($P > 0.05$), whereas lower concentrations and other extracts showed significantly reduced activity ($P < 0.05$) compared to the standard drug. Overall, the results indicate that specific extracts, particularly fruit ethanol, exhibit potent adulticidal activity with efficacy approaching that of the reference anthelmintic.

The anthelmintic efficacy of the tested *C. fistula* extracts

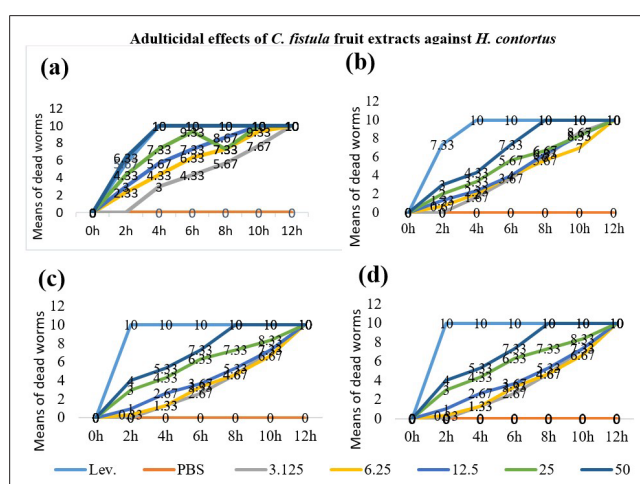


Fig 1. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* fruit extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: (a) ethanol fruits' extract, (b) methanol fruits' extract, (c) ethyl acetate fruits' extract, (d) aqueous-methanol fruits' extract. The values are means of immotile/dead parasites $\pm$ SEM; standard error of mean. The figure is exhibiting the means of dead worms vs the tested time duration of 12 h

Table 2. Phytochemical compounds detected in LC-MS study in different <i>C. fistula</i> stem extracts						
Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in stem ethanol extract	Adipic acid	Fatty Acids	1.81	3.70	146.1	6.84
	Beta Asarone	Phenyl propanoids	4.91	2.80	208.3	3.73
	Erucamide	Fatty Acids	5.51	4.01	337.6	2.93
	Luteolin	Flavonoids	5.41	2.71	286.3	2.01
	Quercetin	Flavonoids	5.81	4.42	302.3	1.70
	Parinaric Acid	Fatty acids	6.80	10.9	276.5	2.71
	22E-Stigmasta-5,22-Di-en-3-Ol	Terpenoids	5.51	6.05	412.7	1.17
	Lupa-12,20(29)-Di-en-3-One	Terpenoids	3.99	7.65	422.8	2.90
	4-Methoxy, Cinnamic Acid	Phenolic Acids	2.72	7.80	178.2	1.72
	Kaempferol	Flavonoids	1.41	12.6	286.3	1.65
	n-Hexadecanoic Acid	Fatty acids	1.87	3.98	256.4	4.57
	Barbaloin	Phenols	1.41	3.44	418.5	1.27
	Trimethylglycine	Amino Acids	2.21	3.61	117.2	1.74
	Quinine	Alkaloids	6.80	12.5	324.4	6.23
Bioactive metabolites present in stem methanol extract	Aloe Emodin	Anthraquinone	3.01	10.75	270.3	2.02
	4-Methoxy Cinnamic Acid	Phenolic Acids	1.01	2.12	178.2	2.21
	3-Hydroxy-3,7-Dimethyl-6- Octenedioic Acid	Fatty Acids	2.90	6.81	186.3	2.52
	Quercetin	Flavonoids	1.31	1.51	302.3	1.11
	Lup-20,29-en-28-al, 3 β -Hydroxy	Terpenoids	1.78	6.55	641	1.23
	Erucamide	Fatty Acids	6.13	1.36	337.3	3.94
	Betulin	Terpenoids	7.13	3.22	442.7	6.77
	Kaempferol	Flavonoids	4.30	1.16	286.2	1.79
	Nervonic Acid	Fatty Acids	1.58	2.87	366.6	5.28
	Palmitic Acid	Fatty Acids	1.79	4.05	256.4	5.09
	Abietic Acid	Terpenoids	1.02	1.11	302.5	1.13
	Apigenin	Flavonoids	3.23	6.04	270.3	1.06
Bioactive metabolites present in stem' ethyl acetate extract	α,ω -Dicarboxylic Acid	Fatty acids	2.83	1.05	286.4	4.62
	Epicatechin	Flavonoids	1.07	1.83	290.2	1.11
	4-Hydroxycoumarin	Phenols	4.83	2.38	162.1	1.35
	8-Hydroxyquinoline	Phenols	2.49	3.07	145.2	2.24
	Quinine	Alkaloids	1.30	3.01	324.4	6.75
	Caffeic Acid	Phenols	3.02	1.61	180.2	2.01
	Barbaloin	Phenols	1.40	2.95	418.4	1.04
	Valine	Amino Acids	3.41	1.41	117.1	1.41
	Aloe Emodin	Fatty Acids	1.25	1.31	270.2	7.34
	Betuline	Terpenoids	1.31	1.31	442.7	1.81
	Rhamnetin	Flavonoids	1.33	2.40	316.2	1.36
	Oleanolic acid	Terpenoids	8.61	1.21	456.7	5.92
	Lupa-12,20(29)-Di-en-3-One	Terpenoids	1.11	1.10	422.7	3.21

Table 2. Continue

Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in stem aqueous methanol extract	α,ω -Dicarboxylic Acid	Fatty Acids	1.87	6.77	146.2	2.18
	(-)-Epigallocatechin	Flavonoids	1.64	6.74	458.4	6.01
	3-pyridinol	Phenols	1.12	5.19	95.1	1.02
	Quinine	Alkaloids	2.88	3.77	324.4	1.20
	Valine	Amino Acids	1.77	1.43	117.2	1.31
	Callosobruchusic Acid	Fatty Acids	1.91	2.82	200	2.03
	Aloe Emodin	Fatty Acids	1.07	3.08	270.2	6.68
	Oleanolic Acid	Terpenoids	4.39	8.35	456.7	3.41
	Ferulic Acid	Phenols	1.51	4.45	194.2	1.11
	7-ethoxycoumarin	Phenols	2.71	1.11	190.3	1.20
	Apigenin	Flavonoids	1.53	1.62	270.2	2.01
	3,6,2'-Trimethoxyflavone	Flavonoids	243	1.81	313.1	1.98

RT: Retention time, MW: Molecular weight, abund.: abundance

RT: Retention time, MW: Molecular weight, abund.: abundance

against adult *H. contortus* followed the order: fruits' ethanol extract (4 h) > leaves' ethyl acetate extract (6 h) > fruits' ethyl acetate and methanol extracts = roots' ethanol extract (8 h) > fruits' aqueous methanol extract = leaves' ethanol extract = roots' methanol, ethyl acetate and aqueous methanol extracts (10 h) > stems' methanol, ethanol and ethyl acetate extracts = leaves' methanol extract (12 h). Overall, the fruit extracts demonstrated greater potency than the roots, stem, and leaf extracts. The activity of all extracts increased significantly in a time- and dose-dependent manner ($P < 0.05$), with the highest concentration (50 mg/mL) showing the strongest effect. Moreover, no inhibition of motility ($0.00 \pm 0.00a$) was

detected at 3.125 mg/mL after 2 h of contact.

Effects of *C. fistula* Extracts Against the Eggs of *H. contortus*

In the egg hatch assay, various *C. fistula* extracts inhibited hatching of *H. contortus* eggs significantly compared with PBS and Oxfendazole (Fig. 5, Fig. 6). Fig. 5 depict the inhibitory effects of fruit and root extracts (Fig. 5-a,b) on egg hatching at different concentrations, respectively. While Fig. 6 show the corresponding effects of leaves and stem extracts (Fig. 6-a,b) in comparison with Oxfendazole and PBS controls. A clear dose-dependent inhibition of egg hatching was observed across all extracts. At the highest concentration (50 mg/mL), the ethanol fruit

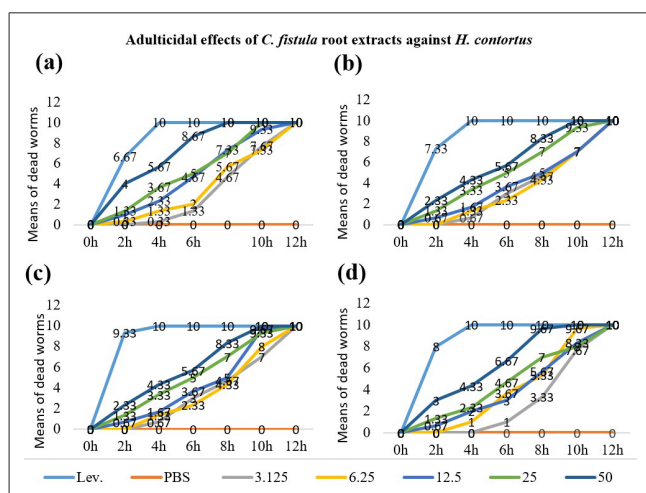


Fig 2. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* root extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: (a) ethanol roots' extract, (b) methanol roots' extract, (c) ethyl acetate roots' extract, (d) aqueous-methanol roots' extract. The values are means of immotile/dead parasites $\pm$ SEM: standard error of mean. Figure is exhibiting means of dead worms vs tested time duration of 12 h

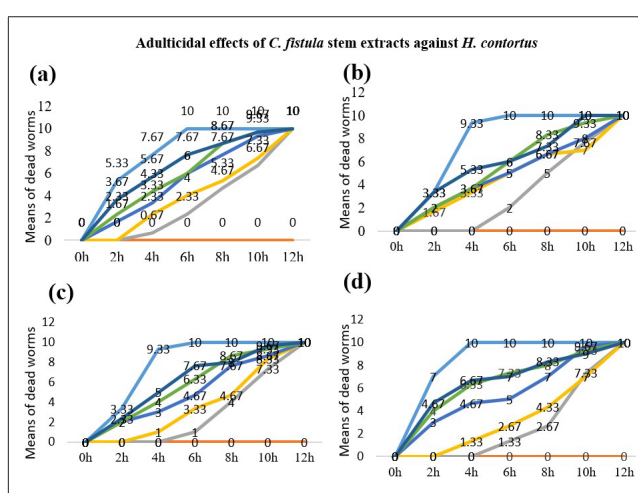


Fig 3. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* stem extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: (a) ethanol stem' extract, (b) methanol stem' extract, (c) ethyl acetate stem' extract, (d) aqueous-methanol stem' extract. The values are means of immotile/dead parasites $\pm$ SEM: standard error of mean. Figure is exhibiting means of dead worms vs tested time duration of 12 h

Table 3. Phytochemical compounds detected in LC-MS study in different <i>C. fistula</i> roots' extracts						
Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in roots' ethanol extract	Mahuannin-D	Phenols	3.81	1.91	528.5	2.29
	Vitispirane	Terpenoids	2.36	2.48	192.2	2.96
	Catechin Hydrate	Flavonoids	1.94	1.64	290.3	2.48
	Lansioside A	Terpenoids	4.11	11.11	659.9	17.3
	Resveratrol	Stilbenoids	2.82	3.85	228.2	2.59
	4O-Methyl melleolide	Sesquiterpenoids	4.66	3.98	414.5	1.76
	Levulinic Acid	Fatty Acids	2.69	1.65	116	2.53
	Protocatechuic Acid	Phenolic Acids	2.66	3.71	154	2.15
	Traumatol	Fatty Acids	5.16	21.17	212.3	21.50
	Gallic Acid	Phenolic Acids	1.71	2.06	170.2	1.54
	Tyramine	Amino Acids	2.08	1.42	137.2	1.56
	p-Coumaric Acid	Phenolic Acids	1.51	1.61	164	2.68
	Catechin	Flavonoids	7.96	5.26	290.3	3.23
	Phytol	Terpenoids	6.51	4.22	296.5	3.67
Bioactive metabolites present in roots' methanol extract	Isoquercitrin	Flavonoids	3.57	1.95	464.5	1.84
	Isoacteoside	Glycosides	1.87	2.15	664.6	1.74
	Gallic Acid	Phenolic Acids	1.54	3.65	170.1	3.11
	Demethoxy curcumin	Polyphenols	1.67	3.55	368.4	3.21
	3,4,5,7,8-Pentamethoxy Flavone	Flavonoids	3.82	2.33	372.4	2.68
	6 β -D-Glucopyranosyl-4,5-Dihydroxy-3,7-Dimethoxy Flavone	Flavonoids Glycosides	1.33	4.43	4773.2	8.66
	Caffeic Acid	Polyphenols	1.91	5.48	180.2	8.95
	Pilosanol A	Flavonoids	6.61	8.03	540.6	8.32
	Tyramine	Amino Acids	2.15	14.6	137.3	7.21
	6-OE-Caffeoylquinic Acid	Terpenoid	1.79	2.87	376.4	3.18
	Senampeline-A	Alkaloids	1.48	1.70	473.5	2.23
	Armillarivin	Terpenoids	2.87	3.18	384.5	2.14
Bioactive metabolites present in roots' ethyl acetate extract	Fluprednisolone	Steroids	3.38	1.45	378.4	1.50
	Rosmarinic Acid	Phenolic Acids	3.78	6.27	360.3	7.54
	Salicylic Acid	Phenolic Acids	2.24	1.97	138.1	1.68
	3R-Hydroxy- β -ionone	Sesquiterpenoids	1.76	7.70	208.3	4.03
	Quercetin,3O-Rhamnoside-7-O-Glucoside	Glycosides	3.08	3.15	610.5	2.17
	Protocatechuic Acid	Phenolic Acids	1.51	8.47	154.1	6.72
	Armillarivin	Terpenoids	1.72	2.28	384.5	3.11
	Epicatechin	Flavonoids	2.11	8.55	290.2	5.03
	B-sitosterol	Phytosterols	1.82	3.77	414.7	4.30
	Luteolin	Flavonoids	3.49	9.76	286.2	8.62
	Catechin	Flavonoids	1.81	1.84	290.3	1.51
	Myricetin	Flavonoids	1.54	2.25	318.2	2.92
	Gallic Acid	Phenolic Acids	2.73	3.86	170.1	5.26
	Silymarin	Flavonoids	1.94	3.55	482.4	4.32

Table 3. Continue

Phyto-Constituents	Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Pantothenic Acid	Vitamins	1.31	4.82	219.2	8.38
Silidianin	Flavonoids	1.28	1.34	482.4	1.40
Astragalin	Flavonoids	1.69	2.72	448.4	1.57
Catechin	Flavonoids	1.26	4.42	290.3	4.05
Rutin	Flavonoids	1.83	2.01	610.5	2.57
6 β -D-Glucopyranosyl-4,5-Dihydroxy-3,7-Dimethoxy Flavone	Flavonoids Glycosides	2.61	1.68	4773.2	2.75
2,6-Di-O-Acetylononin	Phenols	2.54	7.65	514.5	2.77
Myricetin	Flavonoids	1.44	1.51	318.2	1.97
Vanillylmandelic Acid	Phenols	1.74	1.45	166.1	2.33
Gallic Acid	Phenolic Acids	2.86	1.33	170.1	1.39
Perillic Acid	Terpenoids	2.28	5.66	166.2	3.70
Dodecanedioic Acid	Fatty Acids	1.83	2.14	200.3	3.15
4O-Methyl melleolide	Sesquiterpenoids	1.65	3.44	414.5	5.85

RT: Retention time. MW: Molecular weight, abund.: abundance

extract exhibited the strongest inhibitory effect (90.33%), whereas the methanolic root extract showed the lowest activity (56.17%). Statistical analysis revealed that the inhibitory effects of the extracts were significantly higher than the negative control (PBS) ($P < 0.05$). Moreover, the activity of the most effective extracts, particularly the fruit ethanol extract, was comparable to that of the standard drug Oxfendazole ($P > 0.05$), while less active extracts showed significantly lower efficacy ($P < 0.05$). Overall, fruit extracts demonstrated the highest ovicidal activity,

followed by stem, leaf, and root extracts.

The highly potent fruit ethanol extract inhibited 68.33% of eggs hatching even at the lowest tested dose (3.125 mg/mL). At the highest concentration (50 mg/mL), the anthelmintic activity of the various *C. fistula* extracts against *H. contortus* eggs was recorded as follows: ethanol fruits' extract (90.33%), methanol stem' extract

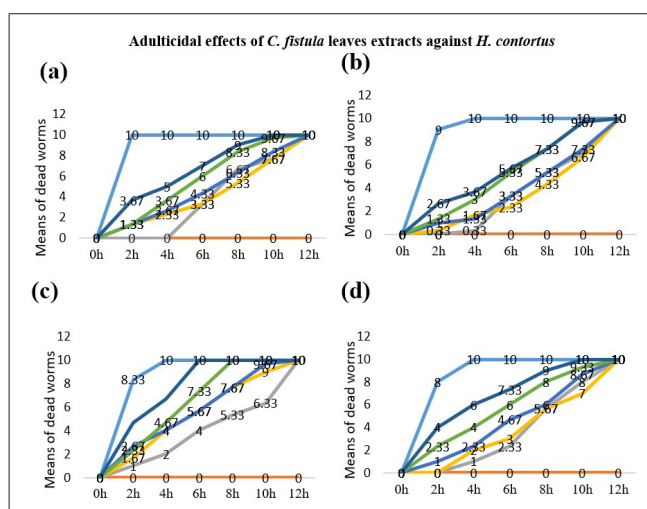


Fig 4. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* leaf extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: (a) ethanol leaves' extract, (b) methanol leaves' extract, (c) ethyl acetate leaves' extract, (d) aqueous-methanol leaves' extract. The values are means of immotile/dead parasites $\pm$ SEM: standard error of mean. Figure is exhibiting means of dead worms vs tested time duration of 12 h

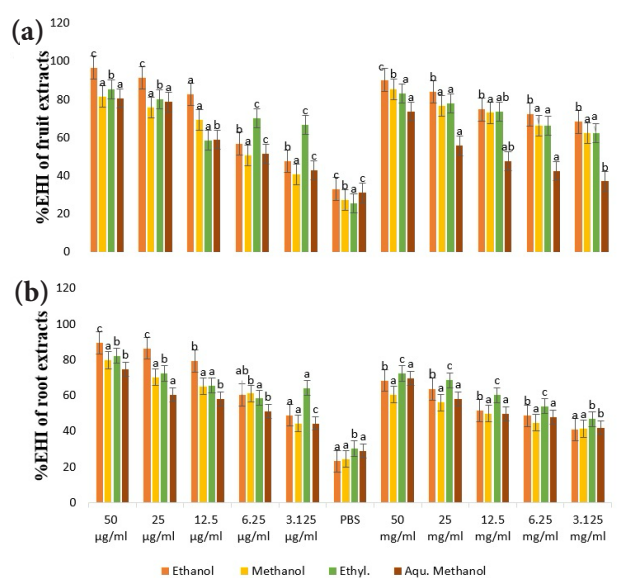


Fig 5. The percentage inhibition of egg hatching against *H. contortus* eggs by (a) *C. fistula* fruit extracts and (b) *C. fistula* root extracts is presented in comparison with various doses of Oxfendazole. Distinct letters (a, b, c) within each column indicate statistically significant differences among the treatment groups. Figure is showing Oxfendazole concentrations in $\mu\text{g/ml}$ and plant extract doses in mg/ml . Ethanol: Ethanol fruit/root extract. Methanol: Methanol fruit/root extract. Ethyl.: Ethyl acetate fruit/root extract. Aqu. Methanol: Aqueous methanol fruit/root extract

Table 4. Phytochemical compounds detected in LC-MS study in different <i>C. fistula</i> fruits' extracts						
Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in fruits' methanol extract	1,1,3-TriethoxyPropane	Esters	2.69	1.18	176.3	5.63
	Caprylic Acid	Fatty Acids	4.16	0.55	144.2	8.71
	6-Chloro-4-Phenyl-2-Propylquinoline	Alkaloids	19.9	23.75	256.7	38.6
	Methyl Salicylate	Esters	5.20	1.44	152.1	4.92
	Decanoic Acid	Fatty Acids	6.74	0.74	200	6.23
	8-Methylnonanoic Acid	Fatty Acids	9.82	5.28	171.2	9.14
	Pentacosane	Alkanes	17.2	0.92	352.7	9.83
	Griseophenone Trimethyl Ether	Benzophenones	17.6	0.02	387.2	5.41
	Pancracine,2O-Dimethyl-	Alkaloids	17.6	0.02	287.3	39.9
	3-Hydroxy-4'-methoxy-6-methylflavone	Flavonoids	18.8	3.95	282	56.62
	4Epi-DehydroAbietinol Acetate	Terpenoids	19.5	0.85	328.5	42.6
	Diocetyl Terephthalate	Esters	18.85	5.71	376.5	46.74
	2-Ethyl-Oxybenzylidene Acetophenone	Flavonoids	19.4	7.29	252.3	78.7
	Antioxidant 425	Phenols	18.1	1.78	340.5	53.42
	N-Heptafluorobutyryl-2-Aminofluorene	Aryl Amines	17.89	0.61	181.2	37.73
	6-Chloro-2,4-Diphenyl Quinoline	Alkaloids	17.89	0.62	315.8	31.54
Bioactive metabolites present in the ethanol extract of fruits	Gallic Acid	Phenolic Acids	8.73	9.82	170.1	27.74
	Beta-Sitosterol Acetate	Terpenoids	14.54	15.31	456.3	31.32
	Trifolin	Flavonoids	18.63	7.84	448.1	21.53
	2,2-DiethoxyPropane	Acetons	17.74	16.82	132.20	20.81
	Corchorifatty Acid F	Fatty Acids	17.52	21.61	328.2	13.32
	n-Hexadecanoic Acid	Fatty Acids	15.64	7.82	256.4	25.53
	4-Epi-DehydroAbietinol Acetate	Terpenoids	19.53	0.85	328.5	34.75
	Stigmasterol	Terpenoids	12.51	9.81	412.37	26.72
	p-Coumaric Acid	Phenolic Acids	13.72	11.52	164	19.95
	Myricetin-3O-Galactoside	Flavonoids	23.511	21.6	480.09	26.73
	6-Chloro-4-Phenyl-2-Propylquinoline	Alkaloids	19.99	23.75	256.6	14.44
	3,4-O-Dimethyl Gallic Acid	Benzoic acids	32.32	25.5	198.05	23.72
	1-Caffeoyl-beta-D-Glucose	Phenolic Acids	11.71	13.5	342.09	17.1
	Di-hydroquercetinGlucosyl Rhamnosy	Flavonoids	29.93	27.3	613.11	22.74
	3O-Caffeoyl-4O-Sinapoylquinic Acid	Phenolic Acids	9.72	11.74	560.3	13.78
Bioactive metabolites present in the aqueous methanol extract of fruits	Dodecanedioic Acid	Fatty Acyls	7.88	16.72	230.2	15.57
	Sinapinic Acid	Phenolic Acids	3.68	3.21	224.2	3.71
	Gallic Acid	Phenolic Acids	7.92	8.74	170.2	7.89
	Isoquercetin	Flavonoids	6.44	7.87	464.2	9.87
	Corchorifatty Acid, F	Fatty Acyls	12	20	328.2	9.72
	Decanoic Acid	Fatty Acids	6.75	0.75	200.1	7.98
	1,1,3-TriethoxyPropane	Esters	2.70	1.19	176.4	4.31
	p_Coumaric Acid	Phenolic Acids	17.7	13.7	164.1	9.83
	4-Propylphenol	Alkylbenzene	8.75	1.73	136.1	7.34
	Syringic Acid	Phenolic Acids	1.09	3.55	194.4	7.71
	Caprylic Acid	Fatty Acids	4.17	0.56	144.2	3.71
	Pyridoxine	Vitamins	18.99	19.38	169.2	21.71
	N-Acetyl-L-Phenylalanine	Aromatic	13.80	14.18	207.2	13.78
	Ethyl Palmitate	Fatty Acids	23.46	24.53	284.5	26.76

Table 4. Continue

Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in fruits' ethyl acetate extract	Prenylated Chalcone	Flavonoids	21.2	23.72	354.4	27.7
	Pentacosane	Alkanes	17.2	0.93	352.7	5.68
	1,1,3-TriethoxyPropane	Esters	20	20.4	182.1	17.2
	2,2-dimethylphenyl 2-chloroacetate	Esters	9.83	5.29	198.3	7.90
	Perillic acid	Terpenoids	7.84	9.82	166.1	10.7
	Isocapric acid	Fatty Acids	9.83	5.29	171.3	8.77
	Gallic acid	Phenolic Acids	5.07	3.19	170.2	9.82
	Octadecane	Alkanes	17.2	7.93	254.5	13.5
	16-Hydroxypalmitic acid	Fatty Acyls	13.7	15.7	272.2	3.72
	Griseophenone, trimethyl ether	Benzophenones	17.6	9.03	387.2	13.1
	n-Propyl acetate	Esters	17.7	13.8	102.2	9.09
	Myricetin-3-Galactoside	Flavonoids	5.07	7.14	480.1	9.09
	Taxifolin 3-O-rhamnoside	Flavonoids	11.1	13.1	613.1	9.20
	Amyrone succinyl	Terpenoids	13.7	9.18	524.5	10.1
	Astragalin	Flavonoids	1.74	3.46	448.1	5.68
	Melilotoside	Aromatics	13.7	13.7	326.1	15.6
	p_coumaric acid	Phenolic Acids	2.09	3.19	164.1	9.20
RT: Retention time. MW: Molecular weight, abund.: abundance						

RT: Retention time. MW: Molecular weight, abund.: abundance

(85.83%), methanol fruits' extract (85.33%), ethyl acetate leaves' extract (83.83%), methanol leaves' extract (83.50%), ethyl acetate fruits' extract (83.33%), aqueous-methanol stem' extract (81%), ethanol leaves' extract (79.17%), aqueous-methanol fruits' extract (73.67%),

aqueous-methanol roots' extract (69.67%), ethanol stem' extract (78.5%), ethyl acetate stem' extract (77.33%), ethyl acetate roots' extract (63.67%), aqueous-methanol leaves' extract (76.17%), and methanol roots' extract (56.17%).

Effect of Ethanol Fruit Extracts and Crude Fruit Powder on Parasite Load in Goats

The *in vivo* anthelmintic activity of *C. fistula* fruit ethanol extract demonstrated the substantial fecal egg count reduction (Table 5). These results illustrate that *C. fistula* holds significant potential for managing gastrointestinal nematode infections in small ruminants. At the dose of 1000 mg/kg b.wt., the goats received ethanol fruit extracts of *C. fistula*, which exhibited the highest efficacy percentage of 90.78% (95% CI), while the goats treated with fruit powder of *C. fistula* exhibited parasites eggs reduction of 70.20%. A single dose oral administration of albendazole (10 mg/kg b.wt.) resulted in an 87.91% reduction in parasite load in the treated goats. Moreover, the efficacy of the ethanol fruit extract revealed an outstanding improvement from the 7th to the 21st day post-treatment. Post-treatment of animals with the fruit ethanol extract of *C. fistula* revealed the highest efficacy in comparison to crude fruit powder and albendazole. There was recorded a significant difference ($P < 0.05$) between animals receiving the ethanol extract and plant powder of *C. fistula* in comparison with the control groups.

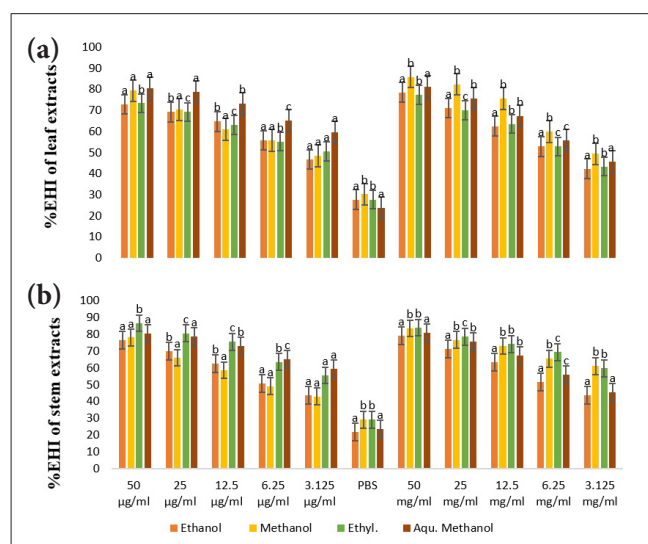


Fig 6. The percentage inhibition of egg hatching against *H. contortus* eggs by (a) *C. fistula* leaf extracts and (b) *C. fistula* stem extracts is presented in comparison with various doses of Oxfendazole. Distinct letters (a, b, c) within each column indicate statistically significant differences among the treatment groups. Figure is showing Oxfendazole concentrations in µg/ml and plant extract doses in mg/ml. Ethanol: Ethanol leaf/stem extract. Methanol: Methanol leaf/stem extract. Ethyl.: Ethyl acetate leaf/stem extract. Aqu. Methanol: Aqueous methanol leaf/stem extract

Table 5. The *in vivo* anthelmintic efficiency of fruit powder and fruit ethanol extract of *C. fistula* against gastrointestinal nematodes in experimentally infected goats as evaluated by faecal egg count reduction assay

Groups	Treatments	% EPG $\pm$ SEM			
		Day 0	Day 7	Day 14	Day 21
T0	No treatment	9.46 $\pm$ 4.09 ^a	8.89 $\pm$ 3.89 ^a	5.39 $\pm$ 3.61 ^a	3.38 $\pm$ 4.07 ^a
T1	ABL 10 mg/kg	43.51 $\pm$ 2.17 ^d	67.61 $\pm$ 1.81 ^e	78.29 $\pm$ 1.71 ^f	87.91 $\pm$ 2.08 ^g
T2	CFE 250 mg/kg b.wt.	9.40 $\pm$ 3.08 ^a	18.75 $\pm$ 3.67 ^b	45.62 $\pm$ 3.71 ^c	74.58 $\pm$ 1.58 ^e
T3	CFE 500 mg/kg b.wt.	8.19 $\pm$ 2.57 ^a	23.99 $\pm$ 3.09 ^{bc}	62.93 $\pm$ 2.21 ^d	79.27 $\pm$ 1.57 ^f
T4	CFE 1000 mg/kg b.wt.	7.15 $\pm$ 3.61 ^a	21.57 $\pm$ 3.29 ^b	59.80 $\pm$ 2.07 ^d	90.78 $\pm$ 0.55 ^g
T5	CFP 250 mg/kg b.wt.	8.29 $\pm$ 3.89 ^a	11.11 $\pm$ 1.46 ^a	33.17 $\pm$ 1.47 ^b	52.54 $\pm$ 1.78 ^c
T6	CFP 500 mg/kg b.wt.	11.37 $\pm$ 3.60 ^a	24.61 $\pm$ 3.09 ^{bc}	40.30 $\pm$ 1.51 ^c	69.53 $\pm$ 1.57 ^d
T7	CFP 1000 mg/kg b.wt.	17.19 $\pm$ 3.08 ^b	34.63 $\pm$ 1.47 ^d	51.21 $\pm$ 1.57 ^{cd}	70.20 $\pm$ 2.51 ^d

Values are means of % EPG $\pm$ SEM: Eggs per gram $\pm$ Standard error of mean. ABL: Albendazole. Day-0 (pre-treatment % EPG). Day 7, 14, 21 (post-treatment % EPG). $P < 0.05$. CFE: *C. fistula* fruit ethanol extract. CFP: *C. fistula* fruit powder

DISCUSSION

The differences observed in extract yield among the various plant parts may be attributed to their distinct biochemical compositions, as certain bioactive compounds exhibit higher solubility in specific solvents than in others [11]. Variations in extract yield are largely influenced by the polarity of the extraction solvents, and differences in biological activity likely reflect variations in the concentration of bioactive constituents being extracted [25]. In this study, the fruit methanol extract (13%) and fruit ethanol extract (12.5%) of *C. fistula* produced the highest yields. The value of *in vitro* assays becomes particularly evident when assessing the effects of plant extracts on multiple developmental stages of parasites: eggs, larvae, and adults, using techniques such as the egg hatch inhibition assays, larval motility inhibition assay, larval exsheathment inhibition test, and adult motility assay [26]. Such multi-stage evaluations help reduce the potential for parasite resistance [27].

In the initial *in vitro* screening, emphasis was on inhibiting egg hatching, especially in helminths exhibiting resistance to benzimidazoles [28]. The egg hatch assay is widely used as a standard approach for assessing the anthelmintic potential of plant-derived compounds [29]. Active phytochemicals can penetrate the eggshell and cause paralysis of L1 larvae. This mode of action not only decreases the parasite's load in the host but also lowers the pasture contamination with infective eggs [30]. The effect of protein fractions from *Combretum leprosum* and *C. fistula* was studied against the larvae and eggs of nematodes infecting goats by Silva et al. [31]. *C. leprosum* exhibited limited inhibition of egg hatching and larval development. *C. fistula* exhibited strong suppressive effects on both stages in naturally infected animals. The egg's inhibitory activity observed in the present study is consistent with the findings reported by Silva et al. [31].

Plant-derived extracts can impair the immune defenses of parasites and induce paralysis by damaging the worm's cuticle [32]. In present study, the strongest anthelmintic activity was observed in the fruit ethanol extract and the leaves ethyl acetate extract at 50 mg/mL, which induced complete paralysis and mortality of adult *H. contortus* (10.00 $\pm$ 0.00), respectively within 4 and 6 hours of exposure, demonstrating activity comparable to the synthetic drug Levamisole. The anthelmintic effects of *C. fistula* against the adult worms of *H. contortus* have not yet been studied. However, the adult motility effects of *C. fistula* in current research work present a close correlation with the anthelmintic effects of *C. alata*; belong to the same family as *C. fistula* [33]. *C. alata* exhibited significant ovicidal and vermifugal effects against the eggs and adults of *H. contortus* upon 6 h exposure to its freeze-dried hydro-acetonic and aqueous extracts [33].

Furthermore, parasite egg counting is essential for determining the *in vivo* efficacy of plant extracts. Increasing the dosage of plant extracts is often justified by the possibility that parasites may develop resistance to the administered compounds, necessitating higher concentrations to achieve the desired therapeutic effect [24]. Plant-derived treatments are generally considered effective when they produce more than a 90% reduction in both adult parasites and eggs per gram (EPG) [34]. According to Reinecke [35], a fecal egg count reduction (FECR) above 90% denotes high efficacy, 80% indicates moderate efficacy, and 60% reflects low efficacy; any FECR below 60% is deemed ineffective. In the present study, the fruit ethanol extract of *C. fistula* at 1000 mg/kg body weight produced the highest FECR of 90.78% (95% CI) indicating high anthelmintic efficacy and representing the most potent treatment among the tested extracts.

In the present study, LC-MS analysis revealed the presence of various bioactive including alkaloids, flavonoids,

amino acids, phenols, alkanes, terpenoids, esters, sterols, fatty acids, and glycosides. *C. fistula* was found to be a rich source of flavonoids, alkaloids, and phenols, and its analysis confirmed the presence of important anti-parasitic constituents, such as quercetin, kaempferol, gallic acid, and hydroxyl-cinnamic acid derivatives (including *p*-coumaric, ferulic, and chlorogenic acids). There is substantial scientific evidence supporting the anthelmintic potential of these pharmaceutical constituents [36]. The presence of key phenolic constituents, such as ferulic acid, gallic acid, vanillic acid, quercetin, and kaempferol, in *Artemisia brevifolia* and the anthelmintic effects of these phyto-components against gastrointestinal nematodes in sheep [37]. The results of the present research show agreement with Hussain et al. [37] and Abdullah et al. [38]. Abdullah et al. [38] studied that the ethanol leaf extract of *C. fistula* contained the highest levels of flavonoids and alkaloids. The presence of these bioactive compounds across different *C. fistula* extracts in the current study indicated their potential as an effective alternative to synthetic drugs for the treatment of haemonchosis and other parasitic diseases.

In conclusion, the anthelmintic activity of various parts of *C. fistula* against *H. contortus* and other gastrointestinal nematodes has been clearly demonstrated. This species belongs to the family *Fabaceae*, contains several anti-parasitic active pharmaceutical ingredients, such as gallic acid, quercetin, kaempferol, and hydroxycinnamic acid derivatives (including *p*-coumaric, ferulic, and chlorogenic acids), which have also been identified and isolated from other members of the same family. Thus, the fruits of *C. fistula* appear to be a rich source of secondary metabolites with significant anthelmintic activity, offering their potential as a promising natural alternative to conventional synthetic drugs for controlling gastrointestinal parasitic infections. Future studies will incorporate coproculture and larval identification to better establish species-specific efficacy and to further align the *in vitro* and *in vivo* components of the work.

DECLARATIONS

Availability of Data and Materials: The data sets generated during the current study, including experimental data from plant extracts and laboratory animals, are available from the corresponding authors (AA & KM) on reasonable request.

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Competing Interests: All authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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RESEARCH ARTICLE

Estimation of Protective Dose 50 of Chromatographically Purified Foot and Mouth Disease Virus Serotype A, Asia 1 and O and Immune Response of Trivalent Vaccine in Bovines

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Abstract

Foot and Mouth Disease (FMD) is an economically significant livestock disease in developing countries such as Pakistan, where serotypes A, Asia1, and O of Foot and Mouth Disease Virus (FMDV) are prevalent. Effective disease control relies on immunization using potent vaccines, and evaluation of vaccine potency in terms of protective dose 50 (PD₅₀) is a critical step during purified vaccine formulation. The present study aimed to prepare chromatographically purified vaccines of the three prevalent serotypes in different formulations and to determine PD₅₀ values. Viruses were propagated in cell culture, biologically titrated, and chemically inactivated. To obtain highly purified antigens with reduced non-structural protein content and improved quantification compared with conventional methods, viral antigens were purified, and quantified using Size Exclusion Chromatography (SEC) with Sephacryl S-300 followed by purity check through SDS-PAGE. Five formulations of each serotype (16, 8, 4, 2, and 1 µg/dose) were prepared and inoculated into five animals per concentration. Serum samples were analyzed by SPCE to identify protected animals. PD₅₀ values were calculated for each antigen and used to formulate a trivalent vaccine. The trivalent vaccine was evaluated for structural and non-structural proteins using SPCE and 3ABC ELISA, respectively. Results demonstrated that SEC efficiently removed NSPs, as confirmed by SDS-PAGE. PD₅₀ values for serotypes A, Asia1, and O were estimated as 2.14, 1.90, and 1.63 µg/dose, respectively. Accordingly, trivalent vaccine concentrations of 6.42, 5.70, and 4.90 µg/dose (3PD₅₀) were formulated. The trivalent purified vaccine induced protective immune responses with less than 20% NSP content in SPCE and 3ABC ELISA.

Keywords: Size exclusion chromatography, Sephacryl S-300, SDS-PAGE, Nonstructural protein, PD₅₀, Vaccine

INTRODUCTION

Pakistan is an agricultural country and livestock (63.6% value addition to agro-sector) serve as the largest subsector contributing 14.97% to national gross domestic product (GDP) during year 2025-2026. More than eight million people are dependent with 35-40% income source from livestock sector. Food in the form of milk, meat & product as well skin & hides are obtained from livestock for the human consumption. It is also the mode of transportation and draught power in rural areas. There is diverse population of animals in Pakistan; buffaloes 47.7M (Million), cattle 59.7M, sheep 33.1M, goat 89.4M & camels 1.2M.

Economic growth, food security and poverty reduction are challenges of developing countries. These are due to the devastating diseases, natural disasters and climate change. Foot and Mouth Disease is one of the World Organization

of Animal Health (WOAH) listed, trans boundary & contagious disease of more than 70 species of animals. The disease is devastating not in term of only production but also economic losses due trade restrictions. Various outbreaks have been reported in neighboring countries due to uncontrolled movements of animals. Pakistan is also facing outbreaks every year in cattle population with heavy economic losses ^[1].

The disease is caused by Foot and Mouth Disease Virus (FMDV), which is positive sense of single stranded RNA member of genus *Aphthovirus* of *Picornaviridae* family. The FMDV is non-enveloped, 146S particle comprised of 8500 bases and small icosahedral protein capsid of 25-30 nm in size made up of four structural proteins each having 60 copies from VP1 to VP3 of 25-34 kDa molecular weight and VP4 of 8-10 kDa ^[2]. Virus produced ten nonstructural



proteins during replication. The FMDV is extremely labile in vitro and converted into its monomers at above 56°C and pH below 6. The FMDV have seven distinct serotypes A, O, Asia1, C, SAT-I, SAT-II and SAT-III [3-5].

Foot and mouth disease can be controlled using various strategies including the forward and backward tracing of infection origin, strict farm quarantines, restriction on movement of animals & their products, identification & culling of infected animals and vaccination of livestock herd. Mass vaccination in FMD endemic areas is key adopted measure by many countries for controlling disease. Inactivated whole culture vaccines are being used to prevent disease. The presence of nonstructural protein (NSP) of FMDV leads to dedifferentiation of vaccinated from infected animals [6,7]. Purified vaccine free from NSPs is required for FMD controlled zone as NSP are produced by infection not by the vaccination. FMD vaccines are produced using baby hamster kidney (BHK-21) cell line. While replication in the cell the FMDV translated its genome into a polyprotein i.e. co- and post-translationally sliced into 14 mature proteins out of which 04 structural protein (SP) and 10 NSPs (Lpro, 2A, 2B, 2C, 3A, 3B1-3, 3Cpro, and 3Dpol), and some cleavage intermediates [8]. Then suspension of virus including cell debris, culture media proteins and NSPs are inactivated with binary ethyleneimine (BEI) or formalin and emulsified with adjuvant to form vaccine. These proteins along with virus produce the immune response and hallmark of differentiation between vaccinated and infected animals. As per WOAHP requirement vaccine must not produce any anti-NSP antibodies after repeated immunization [9].

A potent vaccine formulation depends upon purity and quantity of antigen used. The immunogenicity of FMD vaccines is primarily associated with the presence of intact and purified 146S viral particles [10]. Therefore, concentration and purification of harvested virus are essential steps for efficient storage, quantification of antigen and development of potent vaccines. Various methods, such as precipitation of antigen using polyethylene glycol (PEG) and ammonium sulfate, and ultrafiltration through membrane with particular molecular weight cut off (MWCO) capacity, have been employed for the antigen concentration [11]. For purification and quantification of FMD virus, sucrose density gradient (SDG) ultracentrifugation has traditionally been reference method. This technique separate biomolecules on the basis of size and sedimentation characteristics using linear sucrose gradient approaches [12]. However, SDG ultracentrifugation require specialized equipment and highly skilled, involve lengthy process of 64 h, has high operating and maintenance costs, and has a low sample volume capacity, making it difficult to adopt for vaccine production on an industrial scale [13].

To overcome these limitations Size exclusion chromatography (SEC) has emerged as an alternative approach for purification and quantification of biomolecules [14]. Compared with SDG ultracentrifugation, SEC is a simpler, more economical, and less time-consuming technique that can be readily scaled up by increasing the sample-to-column volume ratio [15]. Furthermore SEC has been successfully utilized for both purification and quantification of FMDV, and has demonstrated a significant correlation value of 0.9669 with SDG-based measurement [16]. The technique utilizes a column packed with porous resin beads where smaller molecules enter pores and elute later, while larger molecules excluded from the pores and elute earlier. These characteristics make SEC a practical and promising method for purification and quantification of FMDV antigen during vaccine production.

Potency is key parameter for evaluating the vaccine that limit the spread of disease. According to European Pharmacopeia (Monograph 04/2005:0063), vaccine potency is determined by measuring the protective dose through challenge studies in animals vaccinated with different antigen payloads. Protective dose 50 (PD₅₀) value of vaccine is well-defined by number of protective doses of antigen which protects fifty percent vaccinated population, when challenged with live virus. Value of PD₅₀ equal or greater than 3 is considered as acceptable for routine vaccination, while equal or greater than 6 is recommended in emergency vaccination in the case of FMD [17,18].

Despite being the gold standard, in-vivo potency test is highly expensive experiment, labor intensive, and associated with ethical concern in FMD endemic countries like Pakistan. These experiments require maintaining high bio-containment animal facilities to safeguard the premises from challenging infection, availability of susceptible host and involve exposure of animals to virulent virus. Furthermore, approximately 50% of unprotected animals may develop painful clinical manifestation of the disease. Even the animals with protective immunity will show lesions at live virus inoculation site and reduced in appetite and weight [19].

Consequently, there is growing interest in adopting substitute techniques that lessen the usage of animals and uphold the 3Rs: replacement, reduction, and refinement [20]. As alternatives to challenge-based potency testing, serological tests like the virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA) have been thoroughly studied. ELISA and VNT significantly lessen animal suffering while estimating the expected level of protection by evaluating antibody responses against virulent pathogen. Potency of FMD vaccine could be estimated indirectly by serological tests capable

of detecting neutralizing antibodies against structural proteins of FMDV [21]. Neutralizing antibodies directed against the structural proteins of FMDV, particularly intact 146S particles, are recognized as an important correlate of protection and can provide valuable information regarding vaccine performance. However, FMD protection is influenced by multiple factors, including antigenic matching between vaccine and field strains, vaccine formulation, adjuvant composition, duration of immunity, and cell-mediated immune responses. Therefore, although serological assays are valuable tools for monitoring immune responses and predicting vaccine efficacy [22], they cannot fully replace challenge-based potency studies, which remain essential for definitive evaluation of FMD vaccine protection.

So therefore, to gain access to international market for the export of FMD free livestock, meat and other commodities, it is important to ensure that vaccines used in FMD control program/campaign should meet the purity requirement with regards to their NSP contents and it contain at least minimum required Protective Doses fifty (3PD₅₀). The study was design to formulate monovalent purified vaccine of local serotype A, Asia1 and O of FMDV with different concentration to estimate PD₅₀ as well as to evaluate anti NSP antibodies.

MATERIAL AND METHODS

Ethical Approval

All animal experiments were reviewed and approved by the Ethical Review Committee, Office of Research Innovation and Commercialization, University of Veterinary and Animal Sciences (approval number: No.DR/157, April 29, 2025), and conducted in accordance with the institutional guidelines for the care and use of laboratory animals.

Sample Collection and Confirmation

Vaccine serotype 'A, Asia 1, O' of FMDV was collected from Quality Operation laboratory (QOL), University of Veterinary and Animals Sciences (UVAS) Lahore. The viruses were confirmed through antigen detection ELISA kit (FMDV antigen and serotyping ELISA, the Pirbright Institute UK, Lot# 01-2021210708a).

Revival of the Cells

Cryopreserved baby hamster kidney (BHK-21) cell line was obtained from Quality Operation Laboratory, University of Veterinary and Animal Sciences. The cells were revived by propagating in Glasgow's Minimal essential medium (GMEM) (Cassion USA) with 10% fetal bovine serum (FBS) at 37°C for 24 h incubation in T-25 flask with 5% CO₂. Next day the cells were disintegrated through trypsin (Gibco, 0.25%) to check the viable count

by dye exclusion method (Trypan blue) and 10⁶ cells/ml were seeded into T-275 flasks and again the incubated at the above condition to develop monolayer. After 24 h cells were checked under inverted microscope for 80-90% monolayer.

Virus Propagation

After the establishment of confluent monolayer (80-90%) in flasks the media was removed. The cells were washed with sterile Phosphate Buffer Saline at least three times. The labeled cells culture flasks were inoculated with filtered FMDV serotype O, A and Asia separately under aseptic conditions for 37°C/40 min to adsorb the virus on cell surface. The flasks were seeded with infection medium (GMEM + 1% FBS) and incubated at 37°C for 24 h (O serotype), 36 h (A & Asia serotype). Following incubation time Cytopathic effects (CPEs) were observed under inverted microscope. After optimal CPEs the flasks were freeze thaw 2-3 time for the release of FMDV from cells. The suspensions containing the FMDV were centrifuge at 1000 rpm/10 min and supernatant were collected and stored at -40°C for further used.

Biological Titration

The stored FMDV serotypes were serially diluted tenfold separately in medium with 1% FBS. Three 96 well plates with monolayer of cells were prepared. The exhausted medium was discarded and each plate was inoculated 100 µL of dilated FMDV in respective wells and up to 10th well while 11 well with pure virus and 12 well with cell control. The plates were incubated at 37°C in CO₂ incubator. Following the incubation CPEs were recorded in 50% as an end point. The biological titer of FMDV serotypes were recorded as a 10_{log} Tissue culture infective dose in samples of FMDV as described by; 50% endpoint titer (TCID₅₀/0.1 mL) = 10_{log} total dilution above 50% + [PD*log (dilution factor)]

Inactivation of Virus

Clarified FMDV were chemically inactivated by using final concentration 3 mM binary ethyleneimine (BEI, MP Biomedical) and 0.04% formaldehyde (MP Biomedical). The residual of inactivation agents were neutralized by 2% Sodium Bis sulphate and sodium thiosulphate for BEI and Formaldehyde respectively. Sample from each inactivated virus were subjected to BHK-21 cells culture flask to check the live viral particle and incubated at 37°C for 24 & 36 h.

Concentration of Antigen Through Polyethylene Glycol (PEG-6000)

Inactivated FMDV serotype A, Asia 1 and O was divided two parts. One part of each serotype was concentrated using PEG-6000 (Deajung, Korea). 100 mL of each virus was inoculated 7.5% PEG from 50% concentrated

solution (Tris Buffer, pH 7.2) and suspension was placed at stirring at 4°C. The suspension was centrifuged and pelleted. Supernatant was discarded and elution of antigen was done using PBS (0.011M, pH 7.2). 1/10 of the original volume of antigen of each virus was obtained and stored at 4°C for further action.

Purification and Quantification of FMDV by Size Exclusion Chromatography (SEC)

The antigen was purified and quantified using BioRad Biologic Chromatograph system 358-BR3506 with UV monitor (2 mm path length) at 254 nm. Biorad Econo Column model 15/50 was filled with resin Sephacryl™ S-300 high resolution (Cytiva, Germany) using PBS 0.011M pH 7.2 and resolution was monitored using solution of Blue dextran (BD) and Bovine Serum Albumen (BSA) with optimized parameter like flow rate (FR = 0.75 mL/min), bed height (48cm), sample volume (W=3.5 mL), path length of flow cell (PL = 0.2 cm), chart recorder speed 12 cm/h (S = 0.2 cm/min). The concentrated sample of each virus was run on chromatographic system and eluted was collected and stored at 4°C for vaccine preparation used. The chromatogram (peaks) of each virus was recorded 254 nm with $E_{cm1\%} = 72$; different sensitivity absorbance unit (FSD) used for the estimation of FMDV in µg/mL using formulae;

$$\text{FMDV in sample (}\mu\text{g/mL)} = \frac{\text{FR} \times \text{PA} \times \text{FSD} \times 1000}{\text{S} \times \text{PL} \times \text{E} \times \text{W}} \quad [23]$$

Purity Confirmation of FMDV

The chromatographic elute of FMDV was run on Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, resolving gel and stacking gel was prepared at concentration 15% & 5% respectively. Sample of chromatographic elute and concentrated was loaded with prestained protein marker (10-180kDa, Servicebio, China) onto well and with Tris-Glycine buffer at 190V for continuous current. The size of band pure and crude FMDV was compared with protein marker.

Vaccines Formulation and Quality Control

Monovalent purified inactivated vaccine of each serotype with different dilution was prepared. For this purpose, 50% of aqueous phase containing quantified contents of 146S fraction of FMDV with 50% of montanide oil ISA 50 (France) as adjuvant was emulsified for each antigen. Five different dilutions of each virus ('A' serotype as A₁ A₂ A₃ A₄ A₅), ('Asia 1' as B₁ B₂ B₃ B₄ B₅) & ('O' as C₁ C₂ C₃ C₄ C₅) at 16, 8, 4, 2 & 1 µg/dose was adjusted with final dose volume 2 mL sterile PBS. Thiomersal sodium was added at 0.01% as preservative. Each vaccine was mixed thoroughly with sterilized homogenizer at room temperature to get consistent vaccine suspension. The prepared purified inactivated vaccine was evaluated for its safety, sterility,

Innocuity and potency testing as described by Razak et al.^[9]. Physical parameters of the vaccines like viscosity, stability and emulsion type were validated as described by Shabana and Abd EL-Sadek ^[24].

Selection of Calves and Inoculation of Vaccines and Serum Collection

A total of seventy-five healthy, adult and unvaccinated bovine calves (above 5 months of age, 25/group, 5/ dilution/vaccine type) of randomly selected breed raised at private cattle farm at FMD free region Bahawalpur, Punjab Pakistan were selected randomly and used for the estimation of potency testing of purified monovalent FMD vaccines. The calves were screened for the presences of antibodies against FMDV type through solid phase competitive ELISA (SPCE). The animals found non protective against FMDV were selected for potency testing and divided into four main group A, B, C & D with each subgroup for each dilution ('A' serotype as A₁ A₂ A₃ A₄ A₅), ('Asia 1' as B₁ B₂ B₃ B₄ B₅) & ('O' as C₁ C₂ C₃ C₄ C₅) and D (Control group, 2/each group). Each group was inoculated with primary dose and following 28 days with booster dose of vaccine from each dilution. All animals of each group were kept under observation for five days post vaccination. Any kind of allergic reaction, edema formation, erythema, changes in body temperature, change in feed intake or any other symptoms were observed. The serum of all animals was collected after 28 days of booster inoculation and tested for the presence of antibodies.

Solid Phase Competitive ELISA (SPCE)

The commercially available blocking ELISA (PrioCHECK™ FMDV Type A, Type Asia 1 and Type O Ab Strip Kit) was used for the detection of antibodies against FMDV. Each ELISA micro plate was coated with non-infectious FMDV type antigen. The reaction between FMDV type and a specific monoclonal antibody (mAb) was blocked by specific antibodies that were present in the test serum of bovine calves and rests of the steps were performed according instruction. For serotype (A, Asia 1), PI = 100-(OD₄₅₀ sample/OD₄₅₀ Max.) *100 (OD₄₅₀ max = control serum (Mean OD ≥1.00)). For serotype O, PI = 100-(Corrected OD₄₅₀ sample/Corrected OD₄₅₀ Max.) *100 (Whereas Corrected OD₄₅₀sample = (OD₄₅₀ sample-Blank Corrected OD₄₅₀Max is control serum) PI<50% is considered negative for FMDV while PI≥50% is considered positive for FMDV.

Determination of Mass Load of FMDV and Estimation of Protective Dose 50 (PD₅₀)

The test sera of animals vaccinated with FMD vaccine having five different concentrations of the 146S fractions for each serotype were declared protective or non-protective based upon the percent inhibition of solid

phase competition ELISA. The minimum concentration of the 146S fraction which elicits 100% protective immune response in calves was determined. Apart from this number of protective doses 50 (PD₅₀) of monovalent FMDV vaccine containing minimum concentration of FMDV Ag but eliciting 100% protection in group of animals based upon SPCE according to WOAHA was estimated as described by Spearman-Kärber's method.

$$PD_{50} (\log_{10}) = - [X_0 - D/2 + D (\sum R/N)]$$

Where, X₀ is log₁₀ of reciprocal of lowest concentration of antigen at 100% protection; D is the log 10 of dilution factor; N is total animals in each group; R is protected out of total animals.

Trivalent Vaccine Formulation and Protective Immune Response

Trivalent vaccine (3PD₅₀) containing purified antigen Serotype A, Asia 1 & O with concentration 6.42 µg, 5.7 µg and 5 µg respectively was formulated with 50% montanide ISA 50 oil adjuvant with standard protocol by adjusting final volume 2 mL/dose. After quality control testing, vaccine was inoculated to five healthy, non-vaccinated calves with primary and booster dose at day 0, and 4th week. Serum was collected at 4th week post booster dose and evaluated for protective antibody titer through Solid Phase competitive ELISA. Anti-NSP antibodies were measured through commercial ELISA kit (IDEXX, 3ABC Bov-Ov). NSP <20% & ≥30% were subjected/considered negative and positive respectively.

Statistical Analysis

The percentage inhibition (PI) values obtained from SPCE ELISA for serotypes A, Asia 1, and O at day 0, 4th week, and 8th week post-vaccination were statistically analyzed by repeated measure ANOVA using SPSS (Version 20, IBM Corp., USA).

RESULTS

Confirmation of Serotypes

FMDV serotyping was confirmed by antigen detection ELISA with positive (≥1.0) and negative control (≤0.1). All the sample were serotyped in their respective well and value for consider to be positive must be greater than 0.1. And for the test validity positive well must be ≥1.0 while negative must be ≤0.1 (Table 1).

Cells Monolayer Development and Propagation of Virus

Cells were revived from stock under aseptic condition and monolayer of cells was first developed in T-25 flask. Following the process the viable cells were counted through dye exclusion method and cells were transferred to T-275 flasks for monolayer developed. After specific incubation

time the 80-90% developed monolayers on specified flask were selected as shown in Fig. 1-A, C, E. Serotype A, Asia 1 & O of FMDV was propagated and incubated at 37°C for 18-24 h & 36 h for O and A, Asia serotype respectively for replication of virus. As virus replicate the specific changes on cells were observed in term of cytopathic effects as shown in Fig. 1-B, D, F). After completion of incubation and observation of CPEs the flasks were freeze thaw for 2-3 time and centrifuge to collect supernatants and stored at -20°C.

Tissue Culture Infective Dose 50 Calculation of Serotype A, Asia 1 and O

Biological titration in term of Tissue Culture Infective Dose 50 (TCID₅₀) for quantification of FMDV serotype A, Asia & O to produce fifty percent cytopathic effects on BHK-21 cells were estimated through reed and munch method (Table 2).

Assessment of Inactivation and Concentration

Inactivated sample of each serotype was inoculated on BHK-21 cells to evaluate the inactivation of the viral antigen. Repeatedly seven blind passages were given to each serotype and CPEs were checked. Up to the 7th

Table 1. serotyping of FMDV through Antigen Detection ELISA

Catching MAbs	Sample	POS Control	NEG Control
Type O	0.545	1.531	0.0332
Type A (1 st Mab)	0.940	1.531	0.0332
Type A (2 nd Mab)	0.031	1.531	0.0332
Type Asia 1	0.297	1.531	0.0332
Type C	0.027	1.531	0.0332
Pan O, A, C & Asia 1	0.517	1.531	0.0332

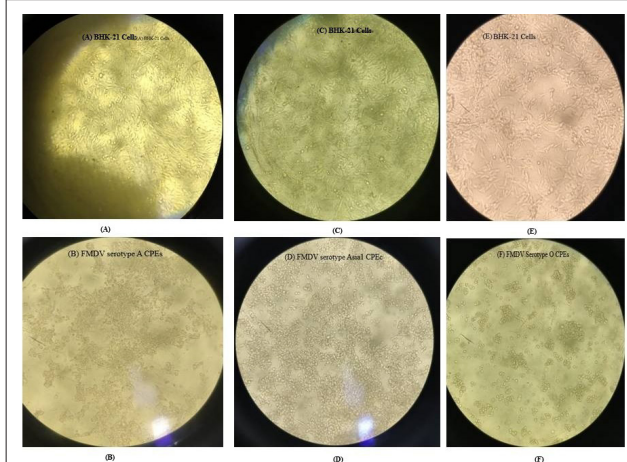


Fig 1. (A) confluent monolayer of BHK-21 Cells, (B) CPEs caused by FMDV serotype 'A' infection (C) confluent monolayer of BHK-21, (D) CPEs caused by FMDV serotype 'Asia 1' infection (E) confluent monolayer of BHK-21, (F) CPEs caused by FMDV serotype 'O' infection

Table 2. Calculation of biological titration (TCID ₅₀)								
Serotype	Dilution	Wells with CPE	Wells without CPE	CP	CN	Total	% CPE	TCID ₅₀ /mL
Serotype A	10 ⁻¹	8	0	46	0	46	100	10 ^{7.29}
	10 ⁻²	8	0	38	0	38	100	
	10 ⁻³	8	0	30	0	30	100	
	10 ⁻⁴	7	1	22	1	23	95.65	
	10 ⁻⁵	6	2	15	3	18	83.33	
	10 ⁻⁶	5	3	9	6	15	60.00	
	10 ⁻⁷	3	5	4	11	15	26.66	
	10 ⁻⁸	1	7	1	18	19	5.26	
	10 ⁻⁹	0	8	0	26	26	0.00	
	10 ⁻¹⁰	0	8	0	34	34	0.00	
Serotype Asia 1	10 ⁻¹	8	0	49	0	49	100	10 ^{7.61}
	10 ⁻²	8	0	41	0	41	100	
	10 ⁻³	8	0	33	0	33	100	
	10 ⁻⁴	8	0	25	0	25	100	
	10 ⁻⁵	7	1	17	1	18	94.44	
	10 ⁻⁶	6	2	10	3	13	76.92	
	10 ⁻⁷	3	5	4	8	12	33.33	
	10 ⁻⁸	1	7	1	15	16	6.25	
	10 ⁻⁹	0	8	0	23	23	0.00	
	10 ⁻¹⁰	0	8	0	31	31	0.00	
Serotype O	10 ⁻¹	8	0	45	0	45	100	10 ^{7.19}
	10 ⁻²	8	0	37	0	37	100	
	10 ⁻³	8	0	29	0	29	100	
	10 ⁻⁴	7	1	21	1	22	95.45	
	10 ⁻⁵	6	2	14	3	17	82.35	
	10 ⁻⁶	5	3	8	6	14	57.14	
	10 ⁻⁷	2	6	3	12	15	20.00	
	10 ⁻⁸	1	7	1	19	20	5.00	
	10 ⁻⁹	0	8	0	23	23	0.00	
	10 ⁻¹⁰	0	8	0	31	31	0.00	

passage no development of CPEs indicated that viruses were inactivated successfully. Following the procedure the 100 mL of each serotype were concentrated 10X (1/10th of original) through PEG-6000 to obtain enough antigens after purification through SEC as it dilutes the sample. About 10ml of each concentrated virus was run on chromatography.

Purification and Quantification Through SEC and SDS-PAGE Analysis

Each concentrated sample was run on chromatographic system and elute was collected. Chromatogram of each virus (Fig. 2-A, B, C) was obtained and peak area was calculated to quantify the virus in sample and eluted in

term of µg/mL in as described in Table 3. Elute of the sample A and Asia 1 was run on SDS-PAGE to check the purity. Structural proteins 64kDa (VP2=VP4), 25kDa (VP3) and 35kDa (VP1) and no bands of non-structural protein at 53kDa (3ABC) and 56kDa (3D) with respect to prestained protein marker (10-180x, Servicebio, China) for purifies FMDV. While in crude sample NSP bands at 53-56kDa along with structural protein were observed (Fig. 3). It is resulted that purified FMDV antigen were free from NSP contents.

Preparation of Monovalent Vaccines

The purified and quantified elute of antigens were diluted to prepared stock of 16 µg/mL of each virus

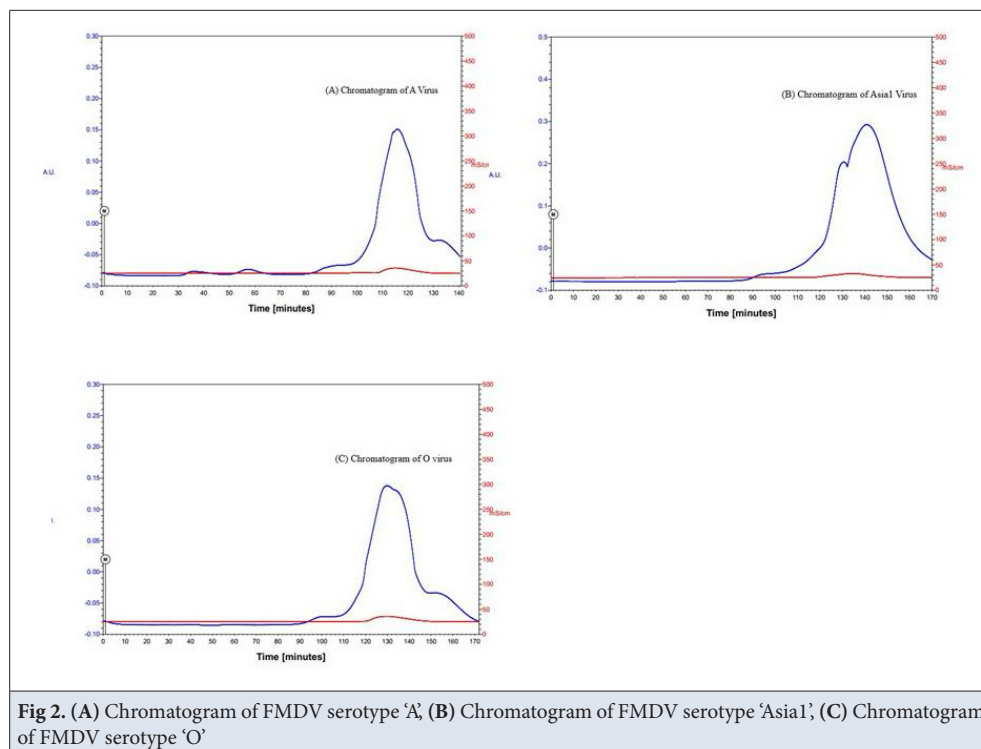


Fig 2. (A) Chromatogram of FMDV serotype 'A', (B) Chromatogram of FMDV serotype 'Asia1', (C) Chromatogram of FMDV serotype 'O'

Table 3. Chromatographic attributes of FMDV serotypes and antigen load ($\mu\text{g/mL}$)

Chromatographic Attributes	Serotype A	Serotype Asia 1	Serotype O
Flow rate of mobile phase (FR)	0.7 mL/min	0.7 mL/min	0.7 mL/min
Sample volume loop (W)	3.5 mL (4% of CV)	3.5 mL (4% of CV)	3.5 mL (4% of CV)
Speed of chart recorder (S)	12 cm/h	12 cm/h	12 cm/h
Extinction point of FMDV ϵ	72	72	72
Path length of flow cell (PL)	0.2 cm	0.2 cm	0.2 cm
Full scale absorbance unit setting (FSD)	0.3	0.5	0.3
Elution volume	28 mL (40 x 0.7)	37.5 mL (54 x 0.7)	37.5 mL (54 x 0.7)
Dilution factor (DF)	$28/3.5 = 8$	$37.5/3.5 = 10.8$	$37.5/3.5 = 10.8$
Length of peak	5.4 cm	5.4 cm	5.0 cm
Base of peak	4.5	5	4.7
Area under peak	12.37 cm^2	13.5 cm^2	11.75 cm^2
Crude concentration * =	= 257	= 468	= 244
Elute Concentration ($\mu\text{g/mL}$) = Crude/Dilution factor	$257/8 = 32.125$	$468/10.8 = 43.75$	$244/10.8 = 22.59$

(Table 4). The purified monovalent vaccines (five concentration of each antigen) were prepared by emulsifying montanide oil ISA 50 and adjusted volume 2 mL/dose (15 doses/30 mL) with sterile PBS as shown in table. Quality testing was performed as per standard protocol.

Vaccine Inoculation and Animal Response

Vaccine of each antigen/concentration was inoculated

to five bovine calves at day 0 & 21st (booster) and blood was collected at different interval day 0, 4th and 8th week of vaccination. Serum was collected and checked through Solid Phase Competitive ELISA for calculation of percentage inhibition (PI) ($\text{PI} \geq 50\%$ protected, $\text{PI} < 50\%$ non-protected) as shown in Table 5. A P-value of less than 0.05 in Mauchly's test of sphericity indicated that the assumption of sphericity was violated, demonstrating significant differences in

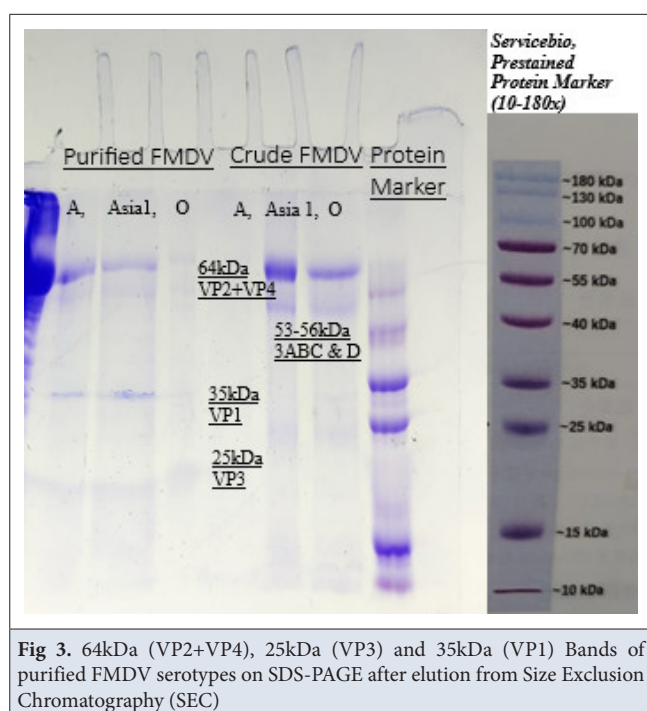


Table 4. Formulation of Purified Inactivated Monovalent FMD Vaccines, against A, Asia 1 and O serotype (Monovalent vaccine for each serotype with same layout)

B #	146S Fraction (µg)/Dose	Ingredients (mL)				Total Volume of Vial/Dose
		Antigen	Montanide Oil	Thiomersal Sodium (µL)	PBS	
B1	16	15	15	3	--	30/15
B2	8	7.5	15	3	7.5	30/15
B3	4	3.75	15	3	11.25	30/15
B4	2	1.875	15	3	13.125	30/15
B5	1	0.9375	15	3	14.065	30/15

percentage inhibition (PI) across day 0, week 4, and week 8. Furthermore, the time $\times$ serotype interaction effect was also statistically significant ($P < 0.05$), indicating that the pattern of antibody response over time differed significantly among serotypes A, Asia 1, and O.

Table 5. PI of SPCE for Serotype A, Asia 1 and O, at day zero, 4th & 8th week

Group A (Serotype A)				Group B (Serotype Asia 1)				Group C (Serotype O)			
Animal Tag No.	Day 0	4 th Week	8 th Week	Animal Tag No.	Day 0	4 th Week	8 th Week	Animal Tag No.	Day 0	4 th week	8 th Week
A ₁₁	41.53	58.78	75.31	B ₁₁	26.52	56.14	82.84	C ₁₁	23.97	55.72	84.57
A ₁₂	31.62	50.95	69.88	B ₁₂	33.15	54.55	79.39	C ₁₂	26.20	51.73	81.41
A ₁₃	40.97	69.72	72.76	B ₁₃	38.19	52.69	88.94	C ₁₃	20.04	53.18	83.64
A ₁₄	44.32	57.58	76.91	B ₁₄	39.25	61.27	87.53	C ₁₄	22.11	52.56	78.51
A ₁₅	41.29	61.42	78.19	B ₁₅	49.04	57.02	88.94	C ₁₅	27.34	50.28	85.60
A ₂₁	36.26	78.99	74.52	B ₂₁	38.28	55.87	83.28	C ₂₁	19.78	53.96	77.68
A ₂₂	30.99	67.41	72.44	B ₂₂	40.40	59.23	90.80	C ₂₂	15.27	53.75	81.67
A ₂₃	33.62	56.94	62.38	B ₂₃	43.85	57.73	87.97	C ₂₃	23.14	53.08	76.44
A ₂₄	36.82	66.21	74.92	B ₂₄	38.01	59.85	90.18	C ₂₄	21.23	52.56	82.08
A ₂₅	38.89	61.74	76.11	B ₂₅	32.36	65.07	82.40	C ₂₅	32.72	50.70	82.81
A ₃₁	35.06	58.14	67.49	B ₃₁	34.30	61.62	81.16	C ₃₁	25.16	53.65	82.96
A ₃₂	21.00	51.27	53.43	B ₃₂	39.08	54.46	85.05	C ₃₂	22.26	37.86	45.83
A ₃₃	32.74	60.14	78.43	B ₃₃	28.20	62.68	88.15	C ₃₃	27.60	50.08	73.54
A ₃₄	38.97	48.80	49.28	B ₃₄	31.83	57.73	86.03	C ₃₄	18.12	50.28	77.01
A ₃₅	28.03	62.85	82.82	B ₃₅	38.90	45.88	48.98	C ₃₅	27.03	54.89	84.41
A ₄₁	14.61	47.36	48.16	B ₄₁	34.92	59.77	84.52	C ₄₁	17.55	56.60	36.30
A ₄₂	25.31	58.22	85.14	B ₄₂	26.17	37.04	38.19	C ₄₂	29.67	51.42	74.31
A ₄₃	47.36	42.65	46.24	B ₄₃	41.73	58.53	86.29	C ₄₃	15.06	52.46	76.44
A ₄₄	8.86	44.16	46.96	B ₄₄	39.52	63.92	85.05	C ₄₄	22.06	27.91	48.99
A ₄₅	41.45	55.43	73.16	B ₄₅	35.19	47.03	48.18	C ₄₅	9.94	51.89	72.14
A ₅₁	32.26	57.90	65.73	B ₅₁	26.79	33.24	36.51	C ₅₁	29.41	37.86	41.22
A ₅₂	23.64	46.40	49.28	B ₅₂	41.99	46.41	46.15	C ₅₂	14.39	53.34	70.17
A ₅₃	34.10	30.35	46.00	B ₅₃	36.25	40.84	40.40	C ₅₃	22.09	51.73	59.14
A ₅₄	25.07	48.00	49.12	B ₅₄	34.74	55.08	68.70	C ₅₄	19.26	23.25	35.53
A ₅₅	28.19	44.48	47.84	B ₅₅	38.19	45.62	47.39	C ₅₅	12.42	27.29	33.66
Control	37.69	38.55	38.01	Control	34.10	35.54	36.02	Control	29.47	35.68	30.50
	38.54	40.31	44.82		38.33	39.88	43.92		33.45	38.58	37.55

Table 6. Estimation of protective dose (PD ₅₀)										
Serotype	G. ID	146S con. (µg/dose)	Dilution	PC	NPC	CPC	CNPC	Total Animals	Protection %	Min. Con. of Ag. (µg/dose) for 50% Protection
Serotype A	A ₁	16	Stock	5	0	17	0	17	100	2.14
	A ₂	8	1:2	5	0	12	0	12	100	
	A ₃	4	1:4	4	1	7	1	8	87.5	
	A ₄	2	1:8	2	3	3	4	7	42.85	
	A ₅	1	1:16	1	4	1	7	8	12.5	
	PD ₅₀ (Log10) = [0 - 0.3010/2 + 0.3010 (12/5)] = 0.5719 = 3.73 PD ₅₀ /dose									
Serotype Asia 1	B ₁	16	Stock	5	0	18	0	18	100	1.90
	B ₂	8	1:2	5	0	13	0	13	100	
	B ₃	4	1:4	4	1	8	1	9	88.8	
	B ₄	2	1:8	3	2	4	3	7	57.14	
	B ₅	1	1:16	1	4	1	7	8	14.28	
	PD ₅₀ (Log10) = [0 - 0.3010/2 + 0.3010 (13/5)] = 0.6321 = 4.21 PD ₅₀ /dose									
Serotype O	C ₁	16	Stock	5	0	19	0	19	100	1.63
	C ₂	8	1:2	5	0	14	0	14	100	
	C ₃	4	1:4	4	1	9	1	10	90.0	
	C ₄	2	1:8	3	2	5	3	8	62.50	
	C ₅	1	1:16	2	3	2	6	8	25.5	
	PD ₅₀ (Log10) = [0 - 0.3010/2 + 0.3010 (14/5)] = 0.6923 = 4.92 PD ₅₀ /dose									
PC: protected calves, NPC: non-protected calves, CPC: cumulative protected calves, CNPC: cumulative non-protective calves										

Estimation of PD₅₀

On the basis of ELISA percentage inhibition interpretation, the animals declared protective (P) and non-protective (NP). Protective Dose 50 for FMDV type A was measure as lowest concentration of antigen (8 µg/dose) give 100 percent protection (PD₅₀ ≥ 3). Likewise, PD₅₀ for Asia 1 and O was determined & describe in Table 6, respectively.

Trivalent Vaccine Preparation, Inoculation and Immune Response

Trivalent vaccine (equal to 3PD₅₀) was prepared by containing antigen concentration (6.42, 5.7 and 4.90 µg/dose) as calculated by estimation of protective dose 50. The five animals were inoculated at day 0 and 4th week (booster dose). Blood was collected at different interval and serum was obtained to check the combined effect of trivalent vaccine and PI was calculated through SPCE. All Animals were protected against three antigens (Table 7). Anti NSP antibodies were checked to assess the purified potential of the vaccine through NSP ELISA (IDEXX, 3ABC Bov-Ov). The entire animal population showed <20% NSP antibodies contend (Table 7).

Table 7. Trivalent vaccine (3PD₅₀) evaluation through SP & NSP ELISA				
Time Interval	Percentage Inhibition for SPCE			
	A	Asia1	O	Control
Day zero	38.18±10.16	37.79±8.16	30.16±6.61	27.11±6.51
4 th week	62.49±4.90	65.96±4.29	57.71±2.25	29.44±6.23
8 th week	79.28±6.95	83.11±7.43	84.97±4.77	32.99±4.42
Percentage for 3ABC NSP				
Day zero	8.83±1.16			
4 th week	8.01±0.77			
8 th week	7.78±1.67			

DISCUSSION

FMD is devastating disease of animals which produced heavy economic losses in term of meat, milk and related products. These products have restriction on export to disease free countries. Potent vaccine is ultimate need to control the disease and spread of live virus. The endemic countries need to check the potency of vaccine to produce NSP free vaccine with DIVA strategy (to distinguish vaccinated from infected animals) to take access to global market for their animal products. To evaluate the potency of the vaccine in-vivo by challenging animals is an

expensive task. Scientists are agreed upon Replacement, Reduction and Refinement (3R) concept to reduce the challenge phase of the testing for animal welfare and cost issues. Alternative methods like challenge free serological approaches are being used with relation of antibody titer to protection level [25].

ELISA OD values for A, Asia1 & O were 0.545, 0.940, and 0.297 respectively (≥ 0.1) [26]. The cells monolayer with 80-90% was developed on flat bottom flasks with inoculation of 10^6 cells/mL at 37°C for 24-36 h. The virus was propagated and after CPEs development virus was harvested and biological titer $10^{7.29}$, $10^{7.61}$, $10^{7.19}$ TCID₅₀ for serotype A, Asia1 & O was calculated using reed and much method. The results were consistent accordance with the study by study [27]. Harvested culture of each serotype virus was double inactivated by BEI and formaldehyde with 3mM and 0.04% respectively followed by ensuring the inactivation process by inoculation on cell culture upto the 7 blind passages. The results are supported by investigation [28].

SEC is efficient technique to purify the 146S content of the FMDV from nonstructural and media protein. Sephacryle S-300 have exclusion limit 10kDa-1500kDa and all the protein from media and NSP are trapped while FMDV (8200kDa) can pass between resin beads; therefore, eluted rapidly [16]. Peak area from UV detector in the form of chromatogram is obtained that is directly related to virus concentration. The optimized condition according to the referenced article [16] were used to calculate the virus concentration 32.125, 43.75 and 22.59 $\mu\text{g/mL}$ for A, Asia1 and O serotype antigen respectively. The result are supported by the study by [16]. Elute was run on SDS-PAGE to check the purity of FMDV with crude virus as a control. Bands of 64, 35, & lighter 25kDa of VP2+VP4, VP1 and VP3 respectively of purified FMDV were seen using 10-180x prestained protein marker. While in crude FMDV the 53-56kDa band of 3ABC & 3D was seen which was absent in the Purified product. These findings are accordance to study which use purified FMDV in SDS-PAGE from SDG method [29].

The vaccine containing 16 & 8 $\mu\text{g/dose}$ of A serotype showed 100% protection while 4, 2 and 1 $\mu\text{g/dose}$ animal group were 87.5, 42.85, & 12.5% respectively. The protective dose in group of animals with 100% percent protection and lowest concentration of virus (8 $\mu\text{g/dose}$) while was 3.73PD₅₀/dose. These are supported by study [9]. Minimum concentration of 2.14 μg was calculated which protect 50% animal in the group. For A serotype 3PD₅₀ = 6.42 $\mu\text{g/dose}$ was estimated in this study. 100% animal protection was observed in vaccine type Asia1 with concentration 16 & 8 $\mu\text{g/dose}$ while remaining 4, 2 & 1 $\mu\text{g/dose}$ were 88.8,

57.14, and 14.28% that mean lowest concentration of 8 $\mu\text{g/dose}$ showed 100% percent protection with PD₅₀ = 4.21/dose. Minimum concentration for 1PD₅₀ was 1.90 $\mu\text{g/dose}$ while 3PD₅₀ = 5.7 $\mu\text{g/dose}$. In the case of O serotype 100% animal protection were calculated in 16 & 8 $\mu\text{g/dose}$; however, the 4, 2, and 1 $\mu\text{g/dose}$ showed the 90.0, 62.5 & 25.5% protection with calculated as 4.92PD₅₀/doses of the lowest concentration of 8 $\mu\text{g/dose}$ (100% protection). Estimated minimum concentration conferring PD₅₀ was 1.63 $\mu\text{g/dose}$ with 3PD₅₀ = 4.89 $\mu\text{g/dose}$. These above findings are accordance with studies [19,30,31] $\geq 3\text{PD}_{50}$ is international requirement for routine vaccine and 6PD₅₀ for emergency vaccination [32].

Trivalent vaccine of FMDV equal to 3PD₅₀ concentration 6.42, 5.7, and 4.90 $\mu\text{g/dose}$ was prepared and inoculated to healthy, with no prior vaccine history of animals (n=5) with primary and booster dose at day zero and 28th week. After 4th week of booster dose serum was collected and checked for SPCE & NSP ELISA. All the animals were protective at week 4 & 8 to three serotypes with mean PI for serotype A; 62.49 $\pm$ 4.90 & 79.28 $\pm$ 6.95, Asia1 65.96 $\pm$ 4.29, 83.11 $\pm$ 7.43 and O 57.71 $\pm$ 2.25, 84.97 $\pm$ 4.7 while NSP contents in all animals were (Mean $\pm$ SD) 8.83 $\pm$ 1.16, 8.87 $\pm$ 0.77, 7.78 $\pm$ 1.67 at day zero, week 4&8 respectively, were less than the cutoff value (20%). Same NSP-ELISA Approach was used and results are accordance with referenced study [33].

In conclusion, present study determined the PD₅₀ values of chromatographically purified FMDV serotypes A, Asia1, and O using serological techniques and evaluated their application in the formulation of a trivalent vaccine in bovines. These findings demonstrate that serological techniques can be alternatively applied for the estimation of PD₅₀ values of purified FMD vaccines, providing a practical approach for vaccine potency assessment and antigen payload standardization in resource setting areas. Furthermore, the study supports the use of SEC as an efficient method for the production of purified FMD vaccines with reduced NSP content and improved consistency for vaccine formulation.

DECLARATIONS

Availability of Data and Materials: The data supporting the findings of this study are available from the corresponding author (I.A.) upon reasonable request.

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Conflict of Interest: The authors declare that they have no relevant financial or non-financial interests to disclose.

Declaration of Generative Artificial Intelligence (AI): The authors declare that no artificial intelligence or AI-assisted technologies were used in the preparation of this article, including the creation of text, tables, or figures except to improve the readability and language of the article.

Author Contributions: FA and IA conceptualize, designed, executed the experiment, compiled the results and wrote the manuscript. ARA analyzed the results. AG interpreted the results, critically revised the manuscript and approved for submission.

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RESEARCH ARTICLE

Cryoprotective and Antioxidant Effects of Maca (*Lepidium meyenii*) Supplementation on Post-Thaw Quality of Ram Sperm

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Abstract

The aim of this study was to evaluate the effects of Maca (*Lepidium meyenii*) supplementation on the freezing process of ram semen. Semen samples were collected from Tuj breed rams using an electroejaculator method and subsequently pooled and divided into four equal volumes. Samples were diluted with a Tris-based extender either without Maca (control) or supplemented with Maca at concentrations of 10 µL (M1), 20 µL (M2), and 30 µL (M3). After freezing, semen samples were thawed at 37°C for 30 sec for analysis. Post-thaw evaluations included sperm motility and kinematic parameters (Computer-Assisted Semen Analysis; CASA), hypoosmotic swelling test (HOST), acrosome integrity, mitochondrial membrane potential (MMP), lipid peroxidation (measured as malondialdehyde, MDA), and intracellular antioxidant status (glutathione, GSH). Data were analyzed using one-way ANOVA and Tukey's post hoc test. After thawing, supplementation with 30 µL of Maca significantly increased total semen motility from 36.14% to 46.21% and progressive motility from 33.56% to 43.91% (P<0.05). Maca, especially at this concentration, also significantly enhanced post-thaw motility, membrane integrity, and antioxidant status compared to the control group (P<0.05). In conclusion, it was determined that Maca added to the semen extender had a positive effect on ram semen.

Keywords: Antioxidant, Maca, *Lepidium meyenii*, Spermatozoa, Cryopreservation, Ram

INTRODUCTION

Sperm freezing and technological methods provide various benefits to animal husbandry, contributing to the preservation of livestock, the conservation of genetic material, and the continuation of its use. However, during cryopreservation, sperm is exposed to oxidative, osmotic, chemical, and physical stress, primarily in the form of ice crystal formation. Consequently, these changes negatively affect sperm quality and fertility [1]. The reason for the negative effect is the increased production of reactive oxygen species (ROS) [2].

High ROS levels cause DNA damage by leading to lipid peroxidation and negatively affect sperm motility and membrane integrity [3]. The role of cryoprotectants is crucial in reducing the stress and problems that occur during cryopreservation. Cryoprotectants aim to reduce increased ROS levels and increase sperm viability and motility [4,5]. Therefore, the selection and amount of

cryoprotectant during cryopreservation is crucial for freezing success.

Plants produce various chemical compounds, including alkaloids, phenolic compounds, and terpenoids. Resveratrol, a good example of these compounds, is a polyphenolic compound used as an antioxidant in humans and domestic animals. Furthermore, these natural metabolites inhibit ROS and also have antimicrobial effects [6]. Therefore, plant extracts are a good alternative to traditional antioxidants in sperm freezing. Maca (*Lepidium meyenii*) is a plant from the Brassicaceae family that grows in the Andes. Its root contains high amounts of sucrose [7] and phytochemicals [8,9]. It also has benefits such as bioactive products, anti-stress antioxidant effects, cytoprotection against natural and oxidative stress, spermatogenesis [10,11], libido in animals and cytoprotection against [12,13] which are closely associated with the upregulation and activity of superoxide dismutase (SOD), a key enzyme in cellular antioxidant defense mechanisms. These effects are



attributed to superoxide dismutase enhancing enzymatic degradation and directly interacting with free radicals [14,15]. Furthermore, Ohta et al. [16] state that Maca may increase the bioavailability of testosterone or testosterone receptors and improve the response of Sertoli cells to follicle-stimulating hormone (FSH), which may have a positive effect on sperm capacity.

Considering the reported bioactive properties of Maca (*Lepidium meyenii*) and its potential influence on oxidative balance and cellular integrity, this study was designed to evaluate the dose-dependent effects of Maca supplementation on ram sperm cryopreservation. The objective is to assess its impact on post-thaw sperm quality parameters using established analytical approaches and to contribute comparable data to the existing body of research.

MATERIAL AND METHODS

Ethical Approval

This study was conducted following the approval of the Animal Research Ethics Committee of Kafkas University (KAÜ-HADYEK/2024-196), Kars, Türkiye.

Animals

Five fertile rams aged between 2 and 4 years old, were used at the Prof. Dr. Ali Rıza AKSOY Education, Research and Application Farm of the Veterinary Faculty of Kafkas University, kept under the same housing, care and feeding conditions.

Preparation of Semen Extender and Aqueous Extract of Maca

Tris-citric acid-fructose-egg yolk-glycerol basic diluent was used in the study [17]. Composition of main diluent are detailed in Table 1 [17]. To obtain a Maca solution, 5 g of Maca powder (commercially available, business registration number:TR-34-K-000495) was mixed with 100 mL of distilled water and stirred automatically in

water bath at 70°C for 3 h. The mixture was centrifuged at 4000 rpm for 10 min and repeated into a water bath at 70°C for 2 h again. The solution was filtrated and stored in a refrigerator at 5°C to use in semen extenders. The preparation and characterization of the Maca powder (including macamides and glucosinolates) and its aqueous extract were based on the methodology described by Del Prete et al. [18], (Table 2) where the extract composition and active compounds were previously defined. Accordingly, the extract used in this study can be considered standardized in terms of its bioactive content. The selected doses were determined based on this reference and were chosen considering their previously reported biological relevance.

Semen Collection

Semen was collected using the electro-ejaculator method twice a week respectively and 10 times in total. The collected sperm was placed in a 32°C water bath and brought to the laboratory, where mass activity (≥ 3 out of 0-5, 5X magnification), motility (70%) (using a phasecontrast (brightfield (A), Ph 2, 40X magnification) microscope (Nikon Eclipse-E400, Tokyo, Japan equipped with a heated slide set at a temperature of 37°C) and sperm concentration 1.5×10^9 (counting was performed under a microscope at 40X magnification using a Thoma slide) assessments were selected for cryopreservation.

Sperm Dilution and Freezing

The pooled semen samples were divided into four equal groups: a control group (C) and three treatment groups supplemented with Maca at concentrations of 10 μ L (M1), 20 μ L (M2), and 30 μ L (M3), and subsequently diluted. The diluted semen samples were drawn into 0.25 mL straws and equilibrated (horizontally) at 4°C for 120 min. Following equilibration, the straws were frozen in liquid

Table 1. Composition of the main semen diluent

Component	Concentration	Function
THAM (Tris; trishydroxymethyl-aminomethane)	27.1 g/L	Buffer; pH stabilization
Citric acid	14.0 g/L	Buffer; ionic balance
D-fructose	10.0 g/L	Energy source
Penicillin G	0.3 g/L	Antibiotic (Gram-positive)
Dihydrostreptomycin	0.4 g/L	Antibiotic (Gram-negative)
Egg yolk	20 % (v/v)	Membrane stabilization; cryoprotection
Glycerol	6 % (v/v)	Cryoprotectant; prevents ice crystal formation

Table 2. Analysis of the powder and maca aqueous extract

Items	Analysis of Maca Types	
	Maca Powder (μ g/L)	Aqueous Extract of Maca (μ g/L)
5-oxo-6E,8E octadecadienoic acid (Macaen)	69.53	17.89
N-(3-hydroxy-benzyl)-2Zfivecarbon acrylamide	614.29	157.99
N-benzyl-5-oxo-6E,8Eoctadecadienamamide (MI 7)	46.08	61.81
N-benzyl-octadecanamamide (MI 16)	53.96	28.89
Macalines or Lepilidines	59.03	13.31
Methyltetrahydro hydridecarboline carboxylic acid	47.17	3.63
1-dibenzyl-2-propane-4,5-dimethylimidazilium		1.25

nitrogen vapour (The temperature range of -100°C to -140°C corresponds to an average value of approximately -120°C) and then transferred to liquid nitrogen for storage until evaluation.

Semen Thawing and Evaluation

The frozen semen samples were thawed at 37°C for 30 s, after which sperm motility and kinematic parameters, the hypo-osmotic swelling test (HOST), acrosome integrity (A), mitochondrial membrane potential (MMP), malondialdehyde (MDA) levels, and glutathione (GSH) activities were analyzed. Sperm motility and kinematic motion parameters were assessed using portable CASA (Androscope, Minitube, D). For the HOST was determined under a phase-contrast microscope (Nikon Eclipse E400, JP). HOS test was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 40 μL of semen with 960 μL of a 100 mOsm/L hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min ^[19].

Flow Cytometric Analysis

The analysis was performed using Attune NxT Acoustic Focusing Cytometer (Invitrogen, USA). The fluorescein isothiocyanate-conjugated peanutagglutinin (PNA)/propidium iodide (PI) dual-staining technique was used to evaluate A. The fluorescence was quantified using a 480 nm excitation wavelength with a 10 nm excitation bandwidth. The emitted light was filtered using a 530/30 nm filter (BL-1) and a 695/40 nm filter (BL-3). The measurements were recorded using Attune NxT software v2.7 (Thermo Fisher, USA). Following the utilization of forward and side scatter light signals to isolate the cell population, the mean fluorescence intensity of the analyzed sperm cells was quantified. The experiment contained a total of 10.000 sperm cells, with a flow rate of 12.5 $\mu\text{L}/\text{min}$. Rhodamine123/PI was used to evaluate MMP. All flow cytometric analysis is performed as previously described by Önder et al. ^[20].

Biochemical Analysis

Thawed semen samples were subjected to spermatological analyses after centrifugation them at 800 g for 10 min, resulting in the separation of the supernatant. Semen samples were used to measure reduced GSH ^[21] and MDA ^[22] levels in the biological samples obtained by using spectrophotometer (Epoch, Biotek, USA). As an MDA standard, 1, 1, 3, 3- Tetramethoxypropane was used, and the results were reported as nmol/mL protein. GSH analysis, the samples underwent precipitation using a 10% solution of trichloroacetic acid, followed by centrifugation at a speed of 1000 g for 5 min. The reaction mixture consisted of 0.5 mL of semen supernatant, 2 mL

of tris hydroxymethyl aminomethane buffer (0.4 M; pH 8.9), and 0.1 mL of 1,5,5'-dithio-bis-2-nitrobenzoic acid. The solution was maintained at room temperature for a duration of 5 minutes, and subsequently measured at a wavelength of 412 nm using the spectrophotometer.

Statistical Analysis

The statistical analysis was conducted using IBM SPSS version 20 (SPSS 20.0 Windows - SPSS, Chicago, IL, USA). The Shapiro Wilk test was used to assess for normality. The data were shown as mean $\pm$ standard error. The statistical significance of the differences between subdivided groups was determined using one-way ANOVA followed by Tukey. Statistical significance was determined for P values below 0.05.

RESULTS

Total and progressive motility [%] are detailed in [Table 3](#), kinematic parameters [%] are detailed in [Table 4](#), A, MMP, and HOST [%] results are detailed in [Fig. 1](#), and MDA [mM/L] and GSH [mg/dL] results are detailed in [Fig. 2](#).

The final total motility in the control, M1, M2, and M3 groups was 36.14, 36.26, 41.17, and 46.21, respectively, while progressive motility was found to be 33.56, 35.86, 38.95, and 43.91 in the control, M1, M2, and M3 groups, respectively. Groups containing Maca, particularly the M3 group, were significantly different from the control group ($P<0.05$). The M3 group was also significantly different from the LIN and STR parameters in terms of kinematic movements ($P<0.05$). The M3 group also achieved the best results in terms of A (74.24%) and MMP (72.06%) ($P<0.05$). Overall, the Maca groups yielded better results compared to the control group, with the M3 group showing significant differences from all other groups in all parameters ($P<0.05$). Total and progressive motility (%) are detail in [Table 3](#), kinematic parameters (%) are presented in detail in [Table 4](#), whereas the results for A, MMP, and HOST (%) are shown in [Fig. 1](#). The MDA levels and GSH activities (%) are summarized in [Fig. 2](#). The highest MDA level was detected in the control group at 10.76 ± 1.46 , while the lowest level was detected in the M3 group at 2.66 ± 0.33 ($P<0.05$). GSH levels were highest

Table 3. Total and Progressive Motility different thawing groups

Groups	Total Motility (%)	Progressive Motility (%)
C (control)	36.14 $\pm$ 0.03 ^a	33.56 $\pm$ 0.34 ^a
M1 (10 μL maca)	39.26 $\pm$ 0.02 ^b	35.86 $\pm$ 0.35 ^b
M2 (20 μL maca)	41.17 $\pm$ 0.003 ^c	38.95 $\pm$ 0.10 ^c
M3 (30 μL maca)	46.21 $\pm$ 0.008 ^d	43.91 $\pm$ 0.36 ^d

a, b, c, d: Groups with different letters in the sperm trait are statistically different ($P<0.05$)

Table 4. Kinematic parameters in different thawing groups

Groups	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	DCL (μm)	DSL (μm)	DAP (μm)	ALH (μm)	BCF (Hz)	LIN (VSL/VCL)	STR (VSL/VAP)
C (control)	70.78±0.19 ^a	52.60±0.30 ^a	54.19±0.43 ^a	26.22±0.005 ^a	20.77±0.04 ^a	21.37±0.008 ^a	1.08±0.01 ^a	7.10±0.04 ^a	0.78±0.005 ^{ab}	0.92±0.02 ^a
M1 (10 μL maca)	72.56±0.56 ^{ab}	53.69±0.23 ^{ab}	55.65±0.23 ^b	26.63±0.24 ^a	21.38±0.31 ^{ab}	21.53±0.04 ^a	1.12±0.03 ^{ab}	7.72±0.24 ^a	0.77±0.03 ^a	0.95±0.005 ^a
M2 (20 μL maca)	74.29±0.35 ^b	55.17±0.08 ^b	57.01±0.40 ^{bc}	27.75±0.21 ^b	22.04±0.20 ^b	22.02±0.03 ^b	1.18±0.01 ^b	8.60±0.17 ^b	0.86±0.01 ^{ab}	0.95±0.02 ^a
M3 (30 μL maca)	77.28±0.64 ^c	57.11±0.55 ^c	58.11±0.06 ^c	28.96±0.33 ^c	23.02±0.03 ^c	22.99±0.06 ^c	1.17±0.01 ^{ab}	8.95±0.09 ^b	0.87±0.01 ^b	0.96±0.003 ^a

a, b, c, d: Groups with different letters in the sperm trait are statistically different ($P<0.05$). VCL: Velocity of Curvilinear, VSL: Velocity of Straight Line, VAP: Velocity of Average Path, DCL: Distance Curvilinear Line, DSL: Distance Straight Line, DAP: Distance Average Path, ALH: Amplitude of Lateral Head Displacement, BCF: Beat Cross Frequency, LIN: Linearity, STR: Straightness

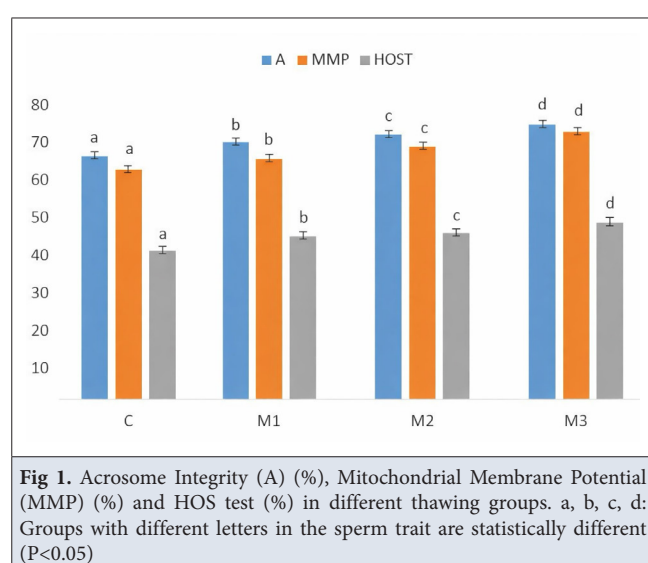


Fig 1. Acrosome Integrity (A) (%), Mitochondrial Membrane Potential (MMP) (%) and HOS test (%) in different thawing groups. a, b, c, d: Groups with different letters in the sperm trait are statistically different ($P<0.05$)

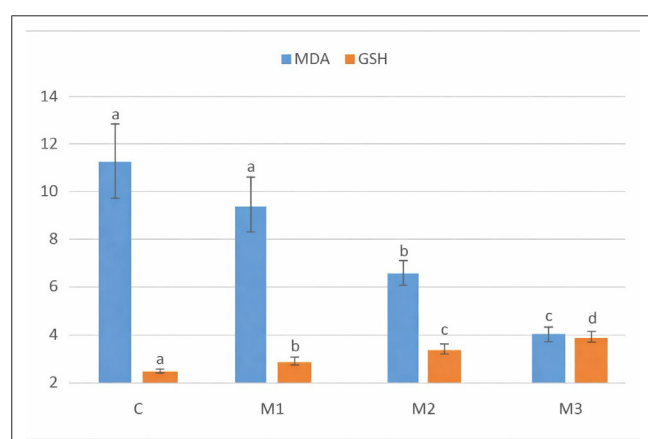


Fig 2. MDA [mM/L] and GSH [mg/dL] parameters in different thawing groups. a, b, c, d: Groups with different letters in the sperm trait are statistically different ($P<0.05$)

in the M3 group at 2.60 ± 0.05 , while the lowest level was found in the control group at 0.36 ± 0.05 ($P<0.05$). The highest values of A, MMP and HOST were determined in the M3 group, with values of 74.24 ± 0.38 , 72.06 ± 0.52 and 47 ± 0.42 , respectively ($P<0.05$).

DISCUSSION

Effective freezing of semen requires understanding the changes that occur during the process, and various studies have been conducted on this subject [23,24]. The primary objective in this process is to preserve and enhance spermatological characteristics during freezing and thawing [25]. In the present study, the aim of this study was to demonstrate whether the addition of Maca to the diluent increases the resistance of semen cells to cryodamage.

Semen motility is the most widely used indicator of semen quality both before and after cryopreservation [26]. It reflects the functional and structural competence of sperm cells [27]. Maca has been reported to improve sperm motility in human semen [28], bovine semen [29], chilled canine semen [18], as well as in experimental studies conducted in mice [30] and stallions [31]. In the present study, Maca supplementation improved sperm motility and kinematic parameters in a dose-dependent manner. Furthermore, one study demonstrated that secondary metabolites of Maca, including macamides, macaenes, Maca alkaloids, and glucosinolates, enhance superoxide dismutase and GSH levels and exhibit iron reducing antioxidant power, hydroxyl radical scavenging activity, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity [32]. These antioxidant properties may explain the beneficial effects of Maca on male reproductive function. Denk et al. [33] examined the effects of quinic acid, a phenolic compound, on ram semen and particularly at 100 and 200 μg, significantly enhanced sperm motility and kinematic parameters, including Prog M, VCL, VAP, and VSL compared to the control. This study consistent with our study, highlighted the compound's positive effects on spermatological parameters. Despite the promising findings of the present study, several methodological limitations should be considered when interpreting the results. Firstly, sperm motility and kinematic parameters evaluated by CASA systems, although widely accepted, may be influenced by system settings, operator variability, and sample handling procedures.

One of the tests performed in our study was the HOST test, which evaluates the integrity of the sperm plasma membrane. This test is considered an important indicator of sperm fertilization capacity [34]. In the current study, HOST rates increased in parallel with the rise in Maca dosage. Leiva-Revilla et al. [28] also reported an improvement in HOST in their study on the effect of Maca on frozen-thawed bovine sperm.

Polyunsaturated fatty acids are found in ram semen including acrosome membranes, mitochondrial membranes, and plasma membranes, all of which are extremely vulnerable to oxidative stress [34]. Oxidative stress increases ROS, leading to increased cell damage and functional losses in sperm [36]. Acrosome integrity is one of the determining factors for fertilization success. Spermatozoa with an intact acrosome can pass through the zona pellucida and fuse with the oocyte [37]. In this study, the addition of Maca to the diluent did not cause damage to the acrosome and mitochondrial membrane and showed therapeutic effects. This suggests that Maca may act as a protective effect on the membrane. Avdatek et al. [38] also reported that 1mM baicalein increases plasma membrane and acrosome integrity in ram sperm.

Malondialdehyde (MDA) is used to assess the intensity of oxidative stress due to lipid peroxidation [39], and GSH is used to determine the effectiveness of the antioxidant defense system [40]. The beneficial effects of Maca on sperm are attributed to the presence of various secondary metabolites such as macaridin, macamicides, Maca alkaloids, and glucosinolates [32]. In our study, the addition of Maca to the semen diluent resulted in a significant difference in MDA and GSH levels, especially in 30 µL. In addition, oxidative stress markers such as MDA and GSH reflect lipid peroxidation and antioxidant capacity; however, they represent only a part of the complex redox balance within sperm cells.

Another limitation is that the study was conducted under *in vitro* conditions, and fertility trials were not performed. Therefore, the positive effects of Maca supplementation on spermatological parameters should be interpreted cautiously, as improvements in laboratory parameters do not always translate into increased fertility outcomes.

In conclusion, Maca supplementation during ram semen cryopreservation showed measurable effects on post-thaw sperm quality, indicating that this plant-derived additive can influence the preservation performance of sperm cells under freezing–thawing conditions. The findings obtained in the present study demonstrate that the response was dose-dependent and that Maca contributed to the maintenance of key spermatological parameters associated with cryopreservation success. Accordingly, the study provides experimentally grounded evidence that Maca may

serve as a biologically relevant supplement in ram semen extenders and offers data that can support the evaluation of antioxidant-based strategies in reproductive biotechnology.

DECLARATIONS

Availability of Data and Materials: Data and Materials are available from the corresponding author (T.G.).

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

Declaration of Generative Artificial Intelligence (AI): The article, tables and figures were not written/ created by AI and AI assisted technologies.

Author Contributions: TG planned the study. TG, MCK and OS performed the semen collection and freezing procedures. NTO performed the semen examination and flow cytometric analyses. LB performed the biochemical analyses. SY and YO ensured the study design, writing and overall control. All authors approved the final version of the manuscript and accept responsibility for the scientific integrity of the work in accordance with the journal's Ethical Principles and Authorship Policy.

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RESEARCH ARTICLE

Sex Ratios of Byzantine Street Dogs in Constantinople: A Zooarchaeological Assessment

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Abstract

Geometric morphometric and fluctuating asymmetry investigations provide robust methodologies for elucidating the living conditions, environmental stressors, and ecological contexts of ancient species using archaeological animal remains. Prior research on Byzantine-era canine skulls discovered at the Theodosius Harbor excavations in Yenikapı, İstanbul, has indicated significant asymmetry in these specimens. This indicates that these dogs were not domesticated companions but rather feral canines (pariahs) contending with severe environmental challenges. This study aims to clarify the demographic composition, namely the sex distribution and sexual dominance, of a largely 'mesocephalic' dog community residing in the urban setting of Byzantine Constantinople. A total of 378 canine skulls from the excavation site were analysed. Sex determination was based on 'basioccipital index' values derived from the length and width measurements of the basioccipital area. The acquired data were subjected to statistical analysis utilizing ANOVA and Kruskal-Wallis tests. Analysis demonstrated statistically significant differences between sexes in the examined population. Among those with determinable sex, the proportion of females (55.6%) surpassed that of males, signifying female predominance. This discovery verifies that dogs in the Byzantine capital encompassed not only working canines under human supervision but also a free-roaming, interbreeding stray population possessing its own social dynamics. The numerical predominance of females indicates their pivotal role in reproductive strategies, facilitating lineage continuity and population sustainability within the demanding urban ecology.

Keywords: Zooarchaeology, Dog, Morphometry, Sex determination, Basioccipital index

INTRODUCTION

Geometric morphometrics, a fundamental technique for assessing morphological variations, facilitates shape analysis in biological contexts ^[1,2]. This approach not only indicates the extent of asymmetry but also elucidates the variation patterns of the analysed structures ^[1].

In morphometric investigations, fluctuating asymmetry a distinct form of asymmetry has been linked to detrimental situations such as stress and hybridization ^[1] and is recognized as a marker of physiological stress ^[3]. Fluctuating asymmetry serves as an indicator of environmental stress ^[4], and its extent generally escalates with heightened environmental stress ^[5-7]. This method has enabled the assessment of cranial asymmetry and its orientation in dogs ^[8]. Research on Byzantine dogs, in

contrast to contemporary domestic breeds, has provided insights about their living standards and conditions ^[9]. The elevated levels of fluctuating asymmetry identified in these investigations were construed as evidence that canines in Constantinople did not inhabit household settings but were, rather, street dogs.

Understanding the status of Byzantine canines is best achieved through the assessment of both literary sources and zooarchaeological evidence. Byzantine sources indicate that dogs were mostly utilized for hunting, herding, and guarding, but there were also guide dogs for the visually impaired and little 'lap dogs' ^[10]. Despite being historically appreciated for their loyalty and proximity to people, religious scriptures and dream interpretations frequently portray them unfavourably as emblems of malevolence, humiliation, and animosity ^[11]. During



crises, this escalated to persecution [10]. Reports indicate that Constantinople accommodated both domesticated dogs and wild animals in its urban environment [10]. Although these animals encountered sporadic persecution in the streets or residences of Constantinople [10], it is well-documented that they were not utilized as a source of meat [10,12]. While dog bones yield significant insights for zooarchaeologists, prudence is necessary when juxtaposing them with contemporary breeds [13]. Regardless of breed comparisons, specific discoveries in archaeological canine remains (e.g., fracture treatments) indicate human influence. These animals, believed to have roamed freely on the streets of Constantinople, occasionally endured hardships during the city's conquests [10]. Substantial evidence for street dogs is derived from a trash site discovered during the Via Carminiello ai Mannesi excavations in Naples, which yielded numerous canine skeletons. It has been proposed that these were probably feral canines pursuing food remnants and rodents [12]. Likewise, canine remains from the Theodosius Harbor region in Constantinople indicate that these were feral animals rather than domesticated pets [9].

This study aims to ascertain the sex distribution and function of these animals, identified as the street dogs of Constantinople [9], inside urban environments. We sought to determine whether male or female dominance was predominant in supporting these generations acclimated to street life. The 'basioccipital index' values [14,15] were utilized for sex determination, with the objective of assessing which sex was more prevalent in interactions with humans.

MATERIAL AND METHODS

Ethics Statement

The materials and methods used in this study were approved by the Muğla Sıtkı Koçman University Local Ethics Committee as not requiring ethical committee approval (E-40051172-050.04-1109624).

The Site and The Background of The Assemblage

This research analysed 378 canine skulls discovered during excavations (2004-2013) at the Yenikapı station, the primary transfer hub for the Metro and Marmaray tunnel on the European side. Excavations verified that this region, referred to as 'Langa Bostanları' during the Ottoman era, was the historical Theodosius Harbor [16], presumably founded by Emperor Theodosius I (AD 379-395) in a cove that extended markedly inland [17]. Excavations in this harbor region uncovered multiple ancient shipwrecks, archaeological artifacts, and animal skeletal remains. The typological classification of the zooarchaeological material revealed that 97% of the dogs were mesocephalic [18]. In addition to this morphometric

study, geometric morphometric analysis of fluctuating asymmetry in the same skulls indicated that these animals were probably street dogs suffering from inadequate dietary conditions [9]. This study was designed to investigate the sexual distribution of street canines and identify the dominant sex in urban environments.

Morphometry of The Skulls

A total of 378 skulls from the Byzantine era were analysed [19]. Sex estimation was conducted utilizing morphological [20] and basioccipital morphometric data [14,15], with the results subjected to statistical analysis. Basioccipital measurements were obtained from each skull to compute the index. A digital caliper with a precision of 1 mm was employed for all measurements. Measurements of the Basioccipital [14,15] (Fig. 1).

Length: A line drawn between the most medial points of the jugular foramina and the Basion (in the median line).

Breadth: Distance between the most medial points of the left and right jugular foramina.

Calculated index [14,15];

Basioccipital index = breadth * 100/length

Investigation of other markings (morphological examination): The condition of the sagittal crest, occipital crest, and basioccipital surface.

Sex Assessment

Basioccipital index values below 123 were categorized as male, whereas those over 136 were categorized as female [15] (Fig. 2, Fig. 3). Values that lie between these two thresholds were categorized as morphologically ambiguous or indeterminate [20].

Statistical Analyses

Statistical analyses of the measurements and derived indices were performed using SPSS 21.0 (Version 21.0,

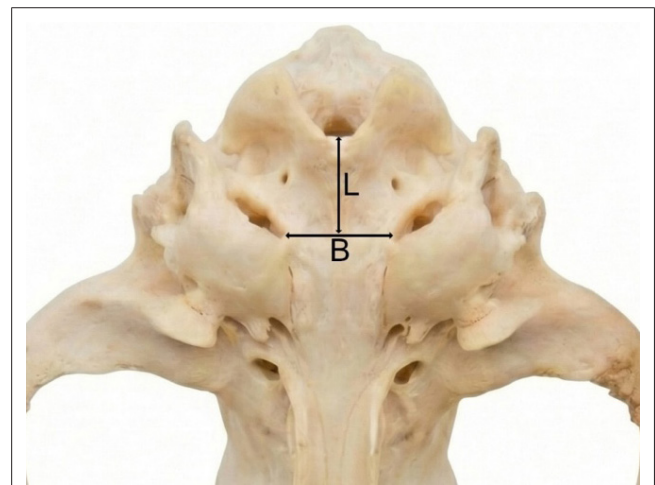


Fig 1. The measurements of the basioccipital region. B. Breadth, L. Length

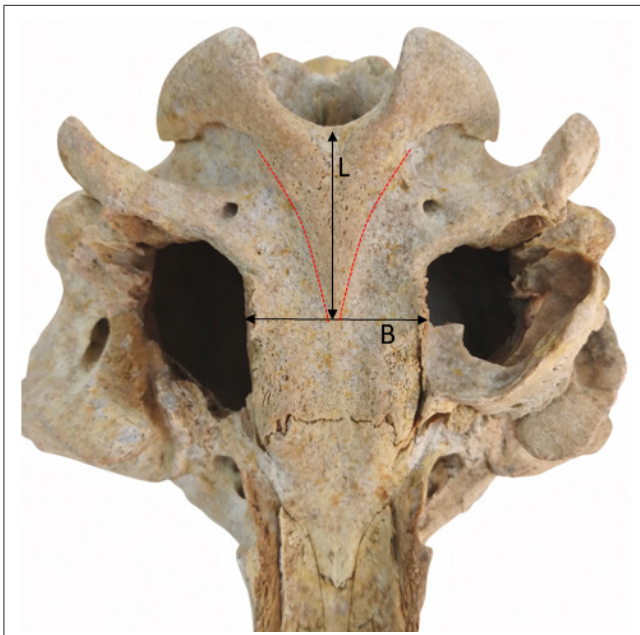


Fig 2. View of the basioccipital bone in male dogs (triangular shape with a pointed apex). B. Breadth, L. Length



Fig 3. View of the basioccipital bone in female dogs (open-ended triangle). B. Breadth, L. Length

SPSS Inc., Chicago, IL, USA). In the analysis of the three groups -those of undetermined sex, females and males- the Kruskal-Wallis test, a non-parametric test, was preferred as the data did not follow a normal distribution. However, in the analysis of the female and male groups, ANOVA was preferred due to the normal distribution of the variances.

RESULTS

Dog skulls were analysed morphologically, although morphometric data constituted the principal foundation

for sex classification. Normality assessments for each group demonstrated that the variables followed a normal distribution. Subsequent to this evaluation, statistical analysis commenced. *Table 1* presents the mean values and standard deviations of morphometric measurements categorized by sex.

The One-Way ANOVA test revealed statistically significant differences between the male and female groups ($P < 0.01$). Post-hoc analyses (Tukey HSD) validated substantial differences among all pairs. Consequently, owing to the presence of non-homogeneous variances, the Kruskal-Wallis test, a non-parametric option, was performed.

The Kruskal-Wallis test indicated significant differences among the three groups concerning the 'Index' variable. This outcome aligns with the preliminary One-Way ANOVA results. The elevated H-statistic (303.800) in the Kruskal-Wallis test signifies a significant disparity across the groups.

Table 2 indicates that 24.6% of the dogs could not be categorized into any sex classification based on morphometric measures. Nevertheless, 75.4% of the skulls conformed to the sex classification ranges delineated in the sex scale [14,15].

Skulls that were not classified by sex, although being morphologically categorized with a predominance of females, were omitted from the morphometric sex determination. Morphological characteristics beyond the index, including the position of the sagittal crest, occipital crest, and basioccipital surface, were assessed.

Sex	Statistical	Length	Breadth
Male	Mean	13.1 ^a	15.3 ^a
	N	75	75
	SD	1.4	1.7
Female	Mean	11.6 ^b	17.2 ^b
	N	210	210
	SD	1.2	1.7
Unidentified	Mean	12.4 ^c	16.2 ^c
	N	93	93
	SD	1.4	1.8

^{a,b,c} Values within a row with different superscripts differ significantly at $P < 0.01$

Sex	N	Mean	SD
Male	75	117.1 ^a	6.0
Female	210	149.2 ^b	10.8
Unidentified	93	130.5 ^c	3.2

^{a,b,c} Values within a row with different superscripts differ significantly at $P < 0.01$

DISCUSSION

Geometric morphometrics, increasingly employed in recent years to assess morphological form variations, facilitates the examination of anatomical structures within biological diversity^[1,21-23]. A significant application of this technology is fluctuating asymmetry. Fluctuating asymmetry, a type of asymmetry that includes directional and antisymmetric, is regarded as a crucial indicator of living conditions, standards, and environmental impacts on biological structures^[1,5,4]. Living environment and related nutrition exert stress on fluctuating asymmetry, leading to heightened asymmetry^[4,9]. Geometric morphometric investigations on dogs have unequivocally illustrated this^[23,24]. Marked disparities in fluctuating asymmetry have been noted between canines residing on the streets and those under human supervision and care. High levels of fluctuating asymmetry in the Byzantine dog skulls examined in this study indicated that these individuals were more susceptible to environmental influences in urban settings^[9]. Byzantine records indicate that, in addition to domesticated dogs, wild or free-roaming animals coexisted inside urban environments^[10]. Typologically, 97% of these Byzantine dogs, as indicated by fluctuating asymmetry investigations^[9], were classified as mesocephalic^[18]. While prior research emphasized the environmental stressors faced by dogs in Constantinople^[9,4], the current study sought to investigate which sex predominantly suited to urban life. The port region in Constantinople, which commenced accumulating alluvium from the Lykos stream in the 7th century^[16,25], was a disposal location for deceased animals until around the 13th century^[26]. The site, recognized as Theodosius Harbor^[16], produced no evidence indicating the eating of urban dogs for meat^[26]. This is corroborated by analogous, though infrequent, Byzantine research^[12,10]. Statistical examinations of the morphometric and index values of these Yenikapı Byzantine dogs reveal considerable differences between male and female individuals. The elevated H-index (303.800) from the Kruskal-Wallis test signified differences. Although each group exhibited a homogeneous distribution internally, considerable differences between sex groups were evident. The 87.5% accuracy rate of sex determination in basioccipital index computations^[14] is notably high. Based on this data, index calculations indicated a greater distribution for females. 55.6% of the skulls were attributed to female individuals. Females outnumbered males in the urban dog population. This indicates a slight female predominance within the social hierarchies of these dogs, which were not categorized as human-fed hunting, herding, or guard dogs^[10]. The morphological analysis of the 24.6% unclassified canines, which predominantly exhibited female characteristics, was excluded from the statistical assessment.

In summary, the sex ratio of street dogs in Byzantine Constantinople was predominantly female. Given that dog remains from the Yenikapı excavations represent around 4% of the total findings^[26], it is clear that canines were integral to social life and that a predominantly female population was present in the city. The predominance of Mesocephalic individuals^[18] indicates that hybrids were prevalent in urban environments rather than canines subjected to human oversight and management. We propose that females had a pivotal role in the reproductive success of these dog lineages.

DECLARATIONS

Availability of Data and Materials: Data and Materials are available from the corresponding author (V.O.).

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Author Contributions: Conceptualization/Resources/Supervision/Project administration VO; Formal analysis, SSS; Investigation, MK, HÖ, VO, WP, SSS, ŞS; Methodology, VO and ŞS; Writing - original draft, VO and ŞS; Writing - review & editing, WP, HÖ, SSS and WP.

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RESEARCH ARTICLE

Prediction of Daily Egg-Laying in Japanese Quails Using Machine Learning

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Abstract

Accurate monitoring of individual productivity in quail farming is essential for maximizing economic returns and shaping effective, sustainable breeding programs. However, daily variation in egg-laying behavior across individuals presents significant challenges to consistent productivity prediction. While conventional methods often struggle to account for these irregular and long-term behavioral trends, machine learning techniques offer a promising alternative through their capacity for individualized, data-driven modeling. In this study, we propose a machine learning-based approach to forecast daily egg-laying outcomes by identifying production patterns from a longitudinal dataset comprising 193 days of continuous observations on 371 quails. The predictive feature set includes rolling averages, cumulative production metrics, and prior-day laying status engineered from each bird's historical production data. Three supervised classification models, Logistic Regression, Random Forest, and Extreme Gradient Boosting (XGBoost) were employed to predict egg-laying on a daily basis. Performance was assessed using accuracy, precision, recall, and F1-score. Among the models, XGBoost outperformed the others, achieving an F1-score of 91.2% and a recall of 97% on the test set. Feature importance analysis identified the 7-day rolling mean as the most influential predictor. These findings underscore the value of machine learning approaches in modeling individual-level laying patterns and demonstrate their potential application in selection decisions, flock-level management, and automated performance monitoring systems in poultry production.

Keywords: Egg-laying prediction, Individual productivity, Machine learning, Precision livestock farming, Quail

INTRODUCTION

The Japanese quail (*Coturnix coturnix japonica*) is a poultry species of considerable economic and scientific importance, widely farmed for its rapid growth, early sexual maturity, and high egg production ^[1,2]. Despite its small body size, the species' prolific laying capacity has made it a valuable model in both commercial production and research settings. In particular, the quail's suitability for confined rearing conditions and efficient feed conversion has positioned it as a promising source of sustainable animal protein, especially in resource-limited contexts ^[3]. Egg production is a key determinant of profitability in quail farming. However, laying performance is shaped not only by genetic factors but also by a range of extrinsic and intrinsic variables, including environmental conditions, nutrition, health status, and individual behavioral tendencies ^[4,5]. This complexity underscores the need for continuous and individualized monitoring systems that can support effective flock management and improve the precision of genetic selection strategies.

Traditional approaches to productivity assessment often rely on weekly or periodic averages. While useful at a flock level, these methods tend to obscure the high variability present in individual laying behavior. They are especially limited in capturing the dynamic, day-to-day fluctuations that are characteristic of long-term production cycles. Accurate modeling of such temporal variability requires analytical tools capable of handling sequential data structures and uncovering subtle behavioral patterns ^[6]. In this context, artificial intelligence -and machine learning (ML) techniques in particular- have emerged as a powerful means of analyzing large-scale, temporally structured data. ML methods have been increasingly applied in animal production systems, including in disease detection, feed intake prediction, and productivity forecasting ^[7]. For example, Ahmad ^[6] compared three neural network architectures for predicting weekly egg production in commercial laying hens, identifying the general regression neural network (GRNN) as the most accurate model. More recently, Lemke et al. ^[8] used



multivariate time-series data to forecast egg production in Hy-Line chickens, applying a sliding window strategy with algorithms such as Ridge Regression, Random Forest, XGBoost, and Multi-Layer Perceptron (MLP). Their findings showed that Ridge Regression, when used with a 7-day window, minimized forecasting errors to as low as 3.81% in mean absolute percentage error (MAPE).

Despite the growing body of literature on ML-based productivity forecasting in poultry, existing studies have predominantly focused on chickens and ducks. To date, there is a conspicuous absence of research applying machine learning to long-term, individual-level daily egg-laying data in quails. This represents a significant gap in the development of precision breeding tools for this species. The present study aims to address this gap by applying a pattern-based machine learning framework to forecast daily egg-laying behavior in Japanese quails. Using a longitudinal dataset comprising 193 consecutive days of egg-laying records from 371 individuals, a range of time-dependent features were engineered -such as 7-day rolling averages, previous-day laying status, and cumulative production. These features were then used to train and evaluate three supervised classification algorithms: Logistic Regression, Random Forest, and Extreme Gradient Boosting (XGBoost). Model performance was assessed using standard metrics including accuracy, precision, recall, and F1-score, with the goal of identifying an effective and generalizable framework for individual productivity modeling in quails.

MATERIAL AND METHODS

Ethical Statement

This study did not involve any procedures requiring ethical approval.

Data Collection and Dataset Description

This study utilizes a longitudinal dataset designed to monitor individual productivity trends in Japanese quails (*Coturnix coturnix japonica*). A total of 371 quails were individually housed under standardized environmental conditions, including controlled lighting, temperature, and feeding regimes. The egg-laying status of each bird was recorded daily over a 193-day period, with egg presence coded as 1 and absence as 0. Missing entries -resulting from mortality or recording errors- were marked as NaN.

The raw dataset was originally structured in a wide format, where rows represented individual birds and columns denoted daily observations. To facilitate temporal analysis, the data were reshaped into a long format, with each row representing a single bird-day observation. This restructuring enabled more flexible feature engineering and temporal modeling.

Feature Engineering

To effectively capture individual productivity dynamics, three temporal features were engineered from each bird's production history:

- RollingMean_7: The 7-day rolling average of egg-laying status, reflecting recent productivity trends
- PrevDay: Binary indicator of egg-laying on the previous day
- CumSum: Cumulative egg count up to the current day

Missing values in predictor variables were imputed using feature-wise median imputation calculated across the dataset, while preserving the overall variance structure of the data. Records containing missing target values or incomplete rolling-window history were excluded from the analysis. In total, approximately 1,200 bird-day observations were excluded during preprocessing. The target for prediction was defined as the egg-laying status (1 or 0) on a given day. After preprocessing, the final dataset comprised over 70,000 valid bird-day observations.

Model Development

Continuous explanatory variables were standardized using z-transformation (mean = 0, standard deviation = 1) prior to model training.

To predict daily egg-laying behavior at the individual level, three supervised machine learning algorithms were applied:

- Logistic Regression (LR)
- Random Forest (RF)
- Extreme Gradient Boosting (XGBoost)

The models were trained to predict the laying status on day $t+1$ using features derived from day t . The dataset was randomly split into training (80%) and test (20%) subsets, with stratification to preserve class distribution. As the class labels (laying vs. non-laying) were relatively balanced, no resampling or weighting adjustments were necessary.

Model performance was evaluated using the following classification metrics:

- Accuracy
- Precision
- Recall
- F1-score
- Area Under the Receiver Operating Characteristic Curve (AUC)

In addition to overall metrics, feature importance scores

Table 1. Comparison of training and test performance metrics of machine learning models

Model	Train Accuracy	Train Precision	Train Recall	Train F1	Test Accuracy	Test Precision	Test Recall	Test F1
Logistic Regression	0.864	0.869	0.944	0.905	0.863	0.868	0.945	0.905
Random Forest	0.881	0.870	0.973	0.918	0.867	0.859	0.965	0.909
XGBoost	0.878	0.866	0.973	0.916	0.871	0.860	0.970	0.912

were extracted for the tree-based models (RF and XGBoost) to interpret predictor contributions. Confusion matrices and ROC curves were also plotted to compare classifier performance visually.

Computational Environment and Software

All analyses were performed in Python 3.9 within a Jupyter Notebook environment on a Linux-based computational server. The following open-source libraries were employed throughout the analysis:

- pandas and numpy for data manipulation and numerical operations
- scikit-learn for model development and performance evaluation
- xgboost for gradient boosting implementation
- matplotlib and seaborn for data visualization

The entire analytical workflow was managed using version control to ensure transparency and reproducibility of data preprocessing and modeling procedures.

RESULTS

The predictive performance of three supervised machine learning algorithms -Logistic Regression (LR), Random Forest (RF), and Extreme Gradient Boosting (XGBoost)- was assessed using daily egg-laying records obtained from 371 individually monitored Japanese quails. The feature set comprised the 7-day rolling average of laying status (RollingMean_7), the previous day's laying outcome (PrevDay), and the cumulative egg count (CumSum). The

dataset was partitioned into training (80%) and testing (20%) subsets to evaluate generalization performance.

All models demonstrated high classification performance on the training data. Logistic Regression achieved an F1-score of 90.5%, serving as a robust linear baseline. Random Forest and XGBoost yielded slightly higher F1-scores of 91.8% and 91.6%, respectively. Notably, Random Forest attained the highest recall (97.3%), indicating strong sensitivity in detecting laying events.

Model performance remained stable on the test dataset, suggesting minimal overfitting. Logistic Regression preserved an F1-score of 90.5%, whereas Random Forest and XGBoost achieved F1-scores of 90.9% and 91.2%, respectively. XGBoost exhibited the highest recall (97.0%) among all models. Precision values were comparable across methods (86-87%), reflecting balanced prediction behavior with controlled false-positive rates (*Table 1*).

Confusion matrix analysis further characterized model behavior. All algorithms effectively classified laying (positive) days; however, Logistic Regression showed relatively lower sensitivity in identifying non-laying (negative) days compared to tree-based models. Random Forest demonstrated a more symmetrical classification profile, achieving high true positive and true negative counts. XGBoost minimized false negatives to the greatest extent, albeit with a marginal increase in false positives (*Fig 1*).

Receiver Operating Characteristic (ROC) analysis produced AUC values of approximately 0.78 for LR and RF, and 0.79 for XGBoost. These results indicate moderate class

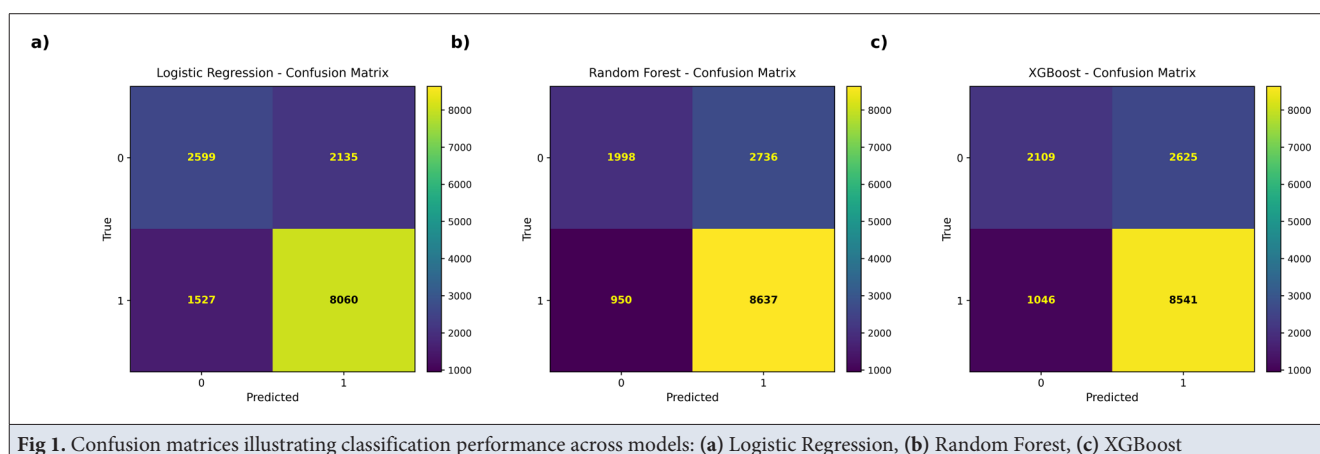


Fig 1. Confusion matrices illustrating classification performance across models: (a) Logistic Regression, (b) Random Forest, (c) XGBoost

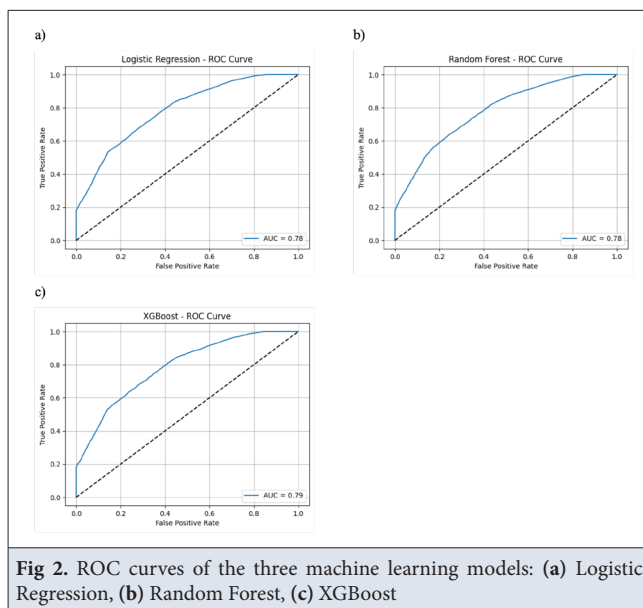


Fig 2. ROC curves of the three machine learning models: (a) Logistic Regression, (b) Random Forest, (c) XGBoost

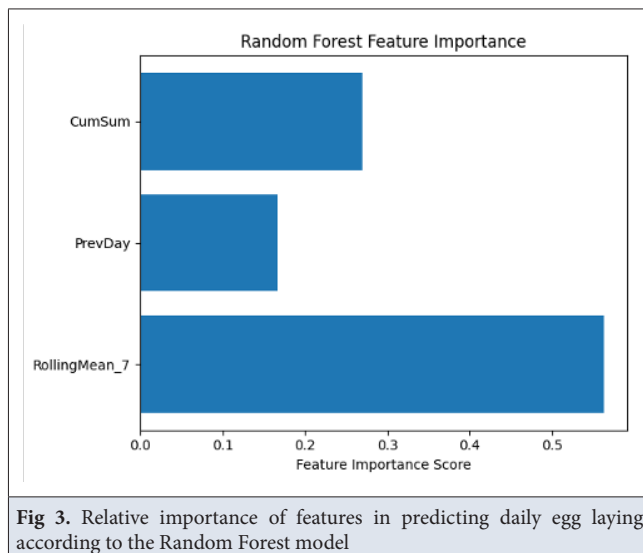


Fig 3. Relative importance of features in predicting daily egg laying according to the Random Forest model

separability despite strong classification metrics (Fig 2).

Feature importance analysis derived from the Random Forest model identified RollingMean_7 as the dominant predictor, followed by CumSum, whereas PrevDay contributed minimally to overall predictive performance (Fig 3).

DISCUSSION

The consistently high predictive performance across all three algorithms underscores the effectiveness of temporal productivity indicators in forecasting daily egg-laying behavior at the individual level. The strong recall values, particularly those achieved by XGBoost, demonstrate the models' capacity to reliably detect productive laying days, thereby reducing the likelihood of overlooking actively laying individuals.

The marginal superiority of tree-based models over Logistic Regression suggests the presence of non-linear interactions within laying behavior patterns that cannot be fully captured by linear decision boundaries. Random Forest provided balanced classification performance, while XGBoost exhibited enhanced sensitivity toward positive events, reflecting its strength in modeling complex feature interactions through gradient boosting mechanisms.

Although the F1-scores and recall values indicate high predictive accuracy, the moderate AUC values (0.78-0.79) suggest that class probability discrimination remains limited. This discrepancy implies that while threshold-based classification performs well, the sharpness of probabilistic separation between classes is comparatively modest. The constrained feature space -restricted to short-term rolling averages, cumulative output, and single-day lag variables- likely contributed to this limitation.

The dominance of RollingMean_7 in feature importance analysis highlights the predictive value of short- and medium-term production dynamics. In contrast, the relatively weak contribution of PrevDay indicates that isolated day-to-day continuity provides limited incremental information beyond aggregated temporal trends. This finding emphasizes that laying behavior is better characterized by dynamic temporal patterns rather than single-point historical indicators.

These observations align with prior research highlighting the significance of temporally structured features in poultry productivity modeling [9,10]. Moreover, the superior recall performance of XGBoost corroborates previous reports advocating gradient-boosted frameworks for minimizing false negatives in livestock and behavioral prediction contexts [11,12]. Comparable advantages of XGBoost have been documented in diverse livestock prediction tasks, including slaughter age estimation in pigs, live weight prediction in sheep, and carcass trait modeling in cattle [13-15], reinforcing its robustness in modeling biologically complex and non-linear systems.

Despite the promising findings, the moderate AUC values suggest that predictive refinement is feasible. Incorporating additional physiological attributes (e.g., age, body weight, health status) and environmental variables (e.g., temperature, photoperiod, housing conditions) may enhance discriminative power. Furthermore, advanced sequence modeling approaches such as long short-term memory (LSTM) networks or attention-based architectures could improve temporal dependency capture and provide deeper insights into individual laying dynamics.

Collectively, these results demonstrate that gradient-boosted machine learning models -particularly XGBoost-

offer a reliable and scalable framework for individual-level egg-laying prediction. By integrating temporal production metrics into predictive modeling pipelines, this approach has the potential to support precision management and genetic selection strategies in quail production systems.

This study demonstrates that individual-level daily egg-laying behavior in quails can be reliably predicted using machine learning models trained on sequential production data. Among the evaluated algorithms, tree-based ensemble methods outperformed the linear baseline, with XGBoost achieving the highest test performance (accuracy: 87.0%, F1-score: 91.2%) and superior recall (97.0%). These results highlight the effectiveness of gradient-boosted frameworks in capturing non-linear temporal production patterns. Feature importance analysis identified the 7-day rolling average (RollingMean_7) as the most influential predictor, emphasizing the dominant role of short-term temporal dynamics over single-day indicators. The findings underscore the value of integrating time-dependent behavioral features into predictive modeling pipelines for precision livestock management. Such approaches enable early detection of productivity shifts and support individual-based monitoring strategies beyond traditional group-level analyses. Future improvements may be achieved through the inclusion of physiological and environmental variables and the application of advanced sequence modeling architectures such as LSTM or attention-based networks. Although conducted in quails, the proposed framework is transferable to other livestock systems with individual-level behavioral data.

DECLARATION

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

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RESEARCH ARTICLE

Molecular Identification of *Anopheles maculipennis* Complex (Diptera: Culicidae) Species in the Northeastern Anatolia Region of Türkiye and Research of *Plasmodium* Presence in These Species

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Abstract

Accurate identification of malaria vectors and detection of pathogens are essential for understanding local transmission dynamics and supporting vector control strategies. In this study a total of 737 adult mosquitoes belonging to the *Anopheles maculipennis* complex (Diptera: Culicidae) were collected from eight ecologically diverse localities in the Aras Valley, northeastern Anatolia, Türkiye, during 2019-2020. Collections were performed using New Jersey light traps and mouth aspirators. Molecular identification was performed using a PCR assay based on ribosomal DNA internal transcribed spacer 2 (ITS2) sequence variation. Electrophoretic profiles and sequence analyses showed that all examined specimens belonged to *Anopheles maculipennis* sensu stricto. This finding indicates that the species is the dominant and widely distributed member of the complex in the study area. To assess the potential for malaria transmission, individual DNA samples were screened for *Plasmodium vivax* and *Plasmodium falciparum* using nested PCR. No *Plasmodium* DNA was detected in any individual analyzed. Although the present findings suggest a low level of malaria transmission risk in the Aras Valley, the extensive distribution of a competent vector species, combined with favorable environmental conditions, highlights the necessity of sustained entomological and molecular surveillance. This study updates molecular data on the *Anopheles maculipennis* complex in northeastern Türkiye and contributes to regional malaria risk assessment.

Keywords: *Anopheles maculipennis* complex, Aras Valley, Malaria, Plasmodium

INTRODUCTION

Malaria has been recognized for centuries as one of the most important vector-borne diseases affecting human populations and continues to represent a major global public health challenge, with an estimated 282 million cases and 610.000 deaths reported worldwide in 2024 ^[1]. Historically, malaria also represented an important public health problem in Türkiye. However, effective control strategies implemented by the Ministry of Health have substantially reduced indigenous transmission, and no locally acquired malaria cases have been reported since 2013. Despite this achievement, Türkiye remains vulnerable to malaria reintroduction because of increasing human mobility, climate change, and the continued presence of competent vector populations ^[2]. Previous reports have suggested that individuals returning from malaria-endemic regions, particularly African countries, as well as international travelers and students, may contribute to the importation of malaria parasites into the country ^[3].

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*, several species of which infect humans. Among these, *Plasmodium falciparum* (Welch 1897), *Plasmodium vivax* (Grassi & Feletti, 1890), *Plasmodium ovale* (Stephens, 1922), *Plasmodium malariae* (Feletti & Grassi, 1889) and *Plasmodium knowlesi* (Knowles and Gupta, 1932) are recognized as the principal causative agents of human malaria. In Türkiye, *P. vivax* has historically been the predominant malaria parasite, whereas *P. falciparum* infections have generally been associated with imported cases. However, recent reports describing indigenous *P. falciparum* infections and mixed *P. falciparum*/*P. vivax* infections suggest a potential influence of increasing international travel and population mobility on malaria epidemiology in the country ^[4,5]. Humans act as intermediate hosts for these parasites, while female *Anopheles* mosquitoes serve as both definitive hosts and vectors.

Among *Anopheles* mosquitoes, the *Anopheles maculipennis* species complex represents one of the earliest



recognized sibling species complexes and includes several epidemiologically important malaria vectors distributed across Europe and the Middle East^[6,7]. Accurate identification of members within this complex has historically been challenging because of substantial morphological similarity among sibling species. Consequently, molecular methods have become increasingly important for reliable species discrimination, whereas traditional approaches such as egg morphology and cytogenetic analyses are now mainly used as complementary tools^[8-12]. Studies focusing on the molecular characterization of the *An. maculipennis* complex in Türkiye have increased particularly since the 2000s^[12-15].

Despite limited data on the vectorial capacities of individual species within the *An. maculipennis* complex, *Anopheles* mosquitoes are recognized as vectors of a range of viral pathogens and filarial parasites, in addition to *Plasmodium* spp. Within the Palearctic region, the *An. maculipennis* complex consists of several closely related sibling species exhibiting considerable ecological diversity and varying vector capacities. To date, only a limited number of members of the complex -*Anopheles maculipennis* s.s. Meigen 1818., *Anopheles sacharovi* Favre 1903, and *Anopheles melanoon* Hackett- have been molecularly confirmed in Türkiye, whereas historical records have also reported *Anopheles messae* Falleroni, 1926, whose taxonomic status remains controversial. These differences among sibling species, particularly regarding ecology, distribution, and vector competence, highlight the importance of accurate species-level identification for disease surveillance and vector control programs.

Understanding the biological and ecological characteristics of vector species is essential for evaluating the transmission dynamics of vector-borne diseases. Furthermore, investigating pathogen presence in mosquito populations contributes to a more accurate assessment of their epidemiological importance and potential role in disease transmission. Although previous studies have investigated mosquito fauna in Türkiye, molecular information regarding the species composition of the *An. maculipennis* complex and the occurrence of *Plasmodium* spp. in northeastern Anatolia remains limited. Therefore, the present study aimed to identify species within the *An. maculipennis* complex collected from the Aras Valley in northeastern Türkiye using molecular methods and to investigate the presence of *Plasmodium* spp. in these mosquitoes. The findings of this study may provide baseline information for future vector surveillance studies and contribute to understanding the potential role of *Anopheles* species in malaria transmission in northeastern Türkiye.

MATERIAL AND METHODS

Ethical Approval

The study does not require ethical committee approval.

Field Studies

In this study, members of the *An. maculipennis* complex were collected from eight localities in the Aras Valley, a region characterized by high potential for vector-borne diseases due to its geographic features, climatic conditions, agricultural and human activities, and availability of mosquito breeding sites. The Aras Valley, which includes the Iğdır Plain, is an important ecological corridor with microclimatic climate features where desert fauna enters Anatolia. Iğdır Province is situated along the southern margin of the Iğdır Plain, at the northwestern foothills of Mount Ağrı. The northern and northeastern boundaries of province are defined by the Aras River, which also forms the border with Armenia, while the eastern and southeastern boundaries are bordered by Nakhchivan and Iran. The study area is characterized by saline soils formed from volcanic materials originating from Mount Ağrı and alluvial deposits transported by the Aras River. The presence of agricultural landscapes, extensive water bodies (including irrigation systems, drainage canals, ponds, and temporary floodwaters), and suitable climatic conditions creates favorable habitats that support high mosquito population densities. Furthermore, the region sustains large populations of livestock, including cattle, sheep, and poultry, which provide abundant blood-meal sources. Collectively, these factors enhance vector-host interactions and may increase the potential for vector-borne disease transmission in the region. According to the 1981-2010 data from the General Directorate of Meteorology, the annual average temperature of the area has been measured at 12.1°C.

The aim was to search for *Plasmodium* parasites in the mosquitoes separated at the species level. Eight different localities (Mürşitalı, Zülfikarköy, Kozlu, Kuloğlu, Yukarı Çıyıklı, Kötek, Sürmeli, and Günindi) (Fig. 1) and their coordinates, as well as the number of mosquitoes collected from these localities are detailed (Table 1).

DNA Isolation

The DNA isolation of the collected samples was performed using the Quick-DNA Tissue/Insect Miniprep Kit (Zymo Research), following the appropriate protocol. The obtained DNA was measured using a spectrometer, and extracted DNA was preserved at -20°C until PCR analysis was performed.

Molecular Identification of the *Anopheles maculipennis* Species Complex

In this study, the identification of the *An. maculipennis*

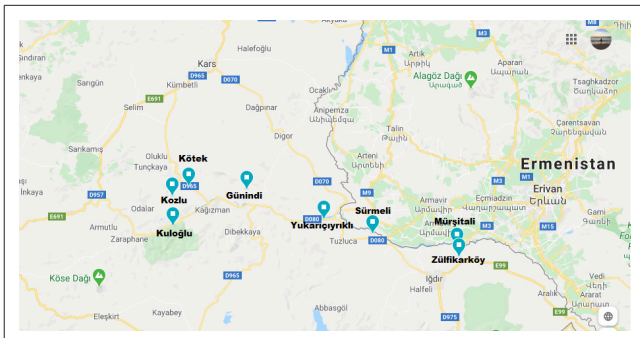


Fig 1. Sampling localities of the evaluated samples

Table 1. Localities where the samples were collected, with their coordinates, elevations, and numbers of samples

Localities	Coordinates	Altitude	Sample Number
Zülfikarköy	39°59'33.3"N 44°08'47.3"E	853 m	133
Mürşitali	40°01'36.7"N 44°08'16.5"E	858 m	90
Kuloğlu	40°11'13.3"N 42°56'59.2"E	880 m	2
Yukarı Çıyırıklı	40°07'31.1"N 43°36'26.4"E	1020 m	276
Sürmeli	40°03'56.9"N 43°47'07.7"E	1020 m	155
Günindi	40°12'24.4"N 43°15'39.6"E	1521 m	3
Kötök	40°13'06.3"N 43°01'07.7"E	1532 m	53
Kozlu	40°11'13.0"N 42°56'59.4"E	2044 m	25
Total			737

species complex was carried out using a method based on differences in ITS2 sequences, which is the most common method for species differentiation. Differentiation based on ITS2 sequence variation was performed following the protocol described by Proft et al.^[16], with additional reference to Akiner and Çağlar^[14]. The following molecular protocol was used to distinguish the *An. maculipennis* complex species.

For the PCR protocol, both general (forward) and species-specific (reverse) primers were used for the species differentiation of the *An. maculipennis* complex. The primers are given in Table 2.

PCR was performed in a 50 µL reaction mixture with 10 pmol of each primer from the seven listed primers, 25 µL of BioMix Red, and DNase/RNase free water. The PCR was conducted under the following conditions: 30 sec at 94°C for denaturation, 30 sec at 55°C for primer annealing, and 30 sec at 72°C for elongation, over 39 cycles.

The PCR products were run on a 1.5% agarose gel at 100 volts for 30 min. After electrophoresis, the species were classified based on band sizes. A 100 base pair marker was used during the electrophoresis process. The band sizes for the species were given in Table 3.

The PCR products were prepared for sequencing and sent for sequencing to obtain raw sequences. After alignment, the sequences were compared to reference sequences in the GenBank database, and species-level matches were identified.

Plasmodium Detection

The previously isolated DNA samples of species complex members, which were identified at the species level used for the detection of *Plasmodium* presence by individually. For this purpose, a two-step nested PCR method was used^[17].

For the initial amplification, a primer pair targeting all four *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) was used to amplify a region approximately 1200 base pairs long. The primers used for this amplification were rPLU5 (5'-CCT GTT GTT GCC TTA AAC TTC-3') and rPLU6 (5'-TTA AAA TTG TTG CAG TTA AAA CG-3'). Positive samples for *P. falciparum* and *P. vivax* were obtained from the Parasitology Bank of the Faculty of Medicine, Celal Bayar University, Manisa. The PCR products obtained from the first step were run on a 1.5% agarose gel, and the presence of bands was checked under UV light.

For the second reaction, PCR was planned to be performed

Table 2. Primers used for species differentiation within the *Anopheles maculipennis* complex

Parameter	Species	Primer
Forward primer	<i>An. maculipennis</i> s.l.	5.8S rDNA (5'-TGT GAA CTG CAG GAC ACA TG-3')
Reverse primers	<i>An. maculipennis</i> s.s.	5'-CGT TTG GCT TGG GTT ATG A-3'
	<i>An. atroparvus</i>	5'-CGT TTG GCT TGG GTT ATG A-3'
	<i>An. messiae</i>	5'-GAC GCC TCA CGA TGA CCT T-3'
	<i>An. melanoon</i>	5'-TGC AAG TTG AAA CCT GGG GC-3'
	<i>An. labranchiae</i>	5'-GTA TCT CTG CTG CTA TGG TC-3'
	<i>An. sacharovi</i>	5'-CAA GAG ATG GAT GTT TTA CG-3'

Table 3. The band size for the *An. maculipennis* complex species

Species	Band Size
<i>An. maculipennis</i> s.s.	410 base pairs
<i>An. atroparvus</i>	117 base pairs
<i>An. messae</i>	305 base pairs
<i>An. melanoon</i>	224 base pairs
<i>An. labranchiae</i>	374 base pairs
<i>An. sacharovi</i>	180 base pairs

with two primer pairs using the positive samples from the first step. For *P. falciparum*, primers rFAL1 (5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3') and rFAL2 (5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3') were used to amplify a region of approximately 205 base pairs. For *P. vivax*, primers (5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3') and rVIV2 (5'-AAG GAA AGA AAG TCC TTA-3') were used to amplify a region of approximately 120 base pairs.

For the first amplification, the PCR-mixture was prepared as follows: 2.5 µL template DNA, 25 µL BioMix Red, and DNase/RNase-free water with 250 nM of each primer (rPLU5 and rPLU6). PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min, with a final elongation at 72°C.

For the second amplification, a total mixture of 20 µL was going to be prepared, and 2 µL of the PCR product from the first amplification was going to be used. However, since no positivity for Plasmodium presence was obtained in the first PCR step, this stage was not conducted.

RESULTS

Molecular Identification of the *Anopheles maculipennis* Species Complex

A total of 737 adult mosquitoes belonging to the *An. maculipennis* complex were collected and the identification method based on the differences in ITS2 sequences of adult individuals carried out using the method described by Proft et al.^[16] as the main reference and the protocol provided by Akiner and Çağlar^[14]. Electrophoresis showed 410 bp bands in all the samples. Sequencing of five randomly selected individuals confirmed that all collected specimens belonged to *An. maculipennis* s.s. Representative gel images showing PCR products of 410 bp are presented (Fig. 2). The forward and reverse sequences obtained from the samples were deposited in the NCBI GenBank database under accession numbers MZ666139–MZ666148.

Plasmodium Detection

For the initial amplification, the first-step PCR was performed using the rPLU5 and rPLU6 primer pairs, which target all four *Plasmodium* species and amplify a region of approximately 1200 base pairs (bp). No *Plasmodium* presence was detected in the first-stage PCR.

Since no positivity was found at this stage, the second-stage PCR was not applied to the samples. The gel image of the nested PCR performed using *P. falciparum* and *P. vivax* positive samples is shown (Fig. 3).

DISCUSSION

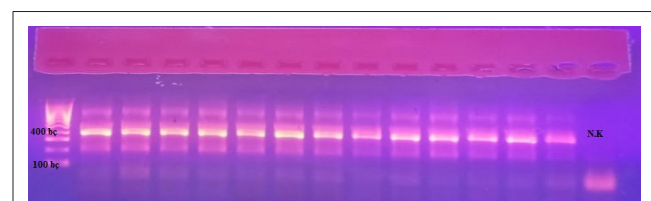


Fig 2. Electrophoresis image of 410 bp agarose gel for *Anopheles maculipennis* s.s.

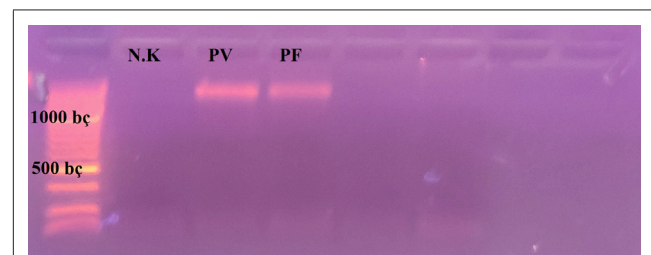


Fig 3. PCR control study performed with *Plasmodium falciparum* and *Plasmodium vivax* positive samples. N.K.: negative control

Effective vector control requires accurate identification of vector species and detailed characterization of their spatial distribution. The Aras Valley was selected as the study area because of its ecological characteristics, including extensive agricultural habitats, irrigation systems, and wetland-associated environments that support mosquito populations. Previous studies have documented medically important mosquito genera, including *Anopheles*, *Aedes*, and *Culex*, in the region^[15,18-23].

Several *Anopheles* species with epidemiological importance in malaria transmission have been reported in Türkiye. Among these, *Anopheles superpictus* Grassi, 1899 and members of the *An. maculipennis* complex, particularly *An. sacharovi* and *An. maculipennis* s.s., are considered epidemiologically important malaria vectors in the Eastern Mediterranean region^[13,24,25].

The *An. maculipennis* complex includes several closely related sibling species that are widely distributed throughout

the Palearctic region and recognized as important malaria vectors in Europe and the Middle East [6,26]. Differences among members of the complex in ecology, geographical distribution, and vector competence have increased the importance of accurate species-level identification. Although the epidemiological roles of individual species are not fully understood, members of the complex have been associated with the transmission of *Plasmodium* spp., arboviruses, and filarial parasites [27]. Previous studies lacking precise species discrimination have reported infections with pathogens such as Batai, Tahyna, West Nile, Usutu, and Sindbis viruses, as well as filarial nematodes [27]. In Türkiye, molecular evidence has confirmed the presence of *An. maculipennis* s.s., *An. melanoon*, and *An. sacharovi*, while *An. messeae* has been reported only in historical records [13,15].

Since its first description, the *An. maculipennis* complex has posed persistent taxonomic challenges because of the high morphological similarity among sibling species. The systematic issues, which started with egg morphology in 1926, are still being addressed with the widespread use of molecular tools. The primary identification method used in our study is the rDNA ITS2 region analysis, which is one of the most commonly used methods in both Türkiye and the Palearctic region. But it is recognized that this method does not provide reliable discrimination between *An. messeae* and *Anopheles daciae* Linton, Nicolescu & Harbach, 2004 [16,28]. In our study, based on the method applied by Proft et al. [16], PCR results yielded bands of approximately 400 bp, and these bands were initially identified as *An. maculipennis* s.s. [16]. Subsequent sequence analyses confirmed that the predominant species detected in northeastern Anatolia belonged to the *An. maculipennis* complex, specifically *An. maculipennis* s.s.

An. maculipennis s.s. is the nominotypical taxon of the species complex and was widely distributed throughout most of Europe, with the exception of its northernmost areas [29]. Recent evidence indicates that the range of this species has extended toward northeastern Europe as well as northwestern Asia and is expanding to the northeast of Europe at a speed of approximately 30 km per year [30]. The establishment of this species in the Southern Urals has been associated with shifts in the composition of sympatric *Anopheles* populations, indicating ongoing changes in species distribution within the Palearctic region [30]. Within the complex, *An. maculipennis* s.s. is distinguished by its ability to occur at elevations exceeding 1,000 m, where larval development generally takes place in small, temporary water bodies associated with agricultural environments [31]. This is consistent with our study, as most of the sampling sites were located above 1,000 m. Despite the detection of *Plasmodium* sporozoites in the salivary glands of this species, its strong

preference for animal hosts indicates a relatively limited role in malaria transmission [32,33]. In a previous study conducted in the Aras Valley, *Dirofilaria immitis* (Leidy, 1856) and *Dirofilaria repens* Railliet & Henry, 1911 were detected in these complex mosquitoes identified only at the genus level [34]. Considering the predominance of *An. maculipennis* s.s. observed in the present study, it is possible that this species was responsible for the previously detected *Dirofilaria* infections.

A large-scale mosquito survey conducted in Armenia between 2016 and 2018 identified *An. maculipennis* s.l./s.s. as the predominant *Anopheles* species in the country, whereas *An. sacharovi* was detected only in low numbers in the Ararat Valley [35]. Similarly, larval sampling studies from northern Iran reported *An. maculipennis* s.l. as the most abundant *Anopheles* species in the Mazandaran region [36]. Consistent with these findings, Günay [15] detected only *An. maculipennis* s.s. in the Iğdır and Kars regions using COI gene sequences and reported sympatric distribution of genetically distinct groups temporarily designated as *Anopheles* MBI-36. Although Kuçlu [22] identified *An. sacharovi* in the Aras Valley based on a limited number of specimens, this species was not detected in the present study, which may reflect differences in sampling methods, seasonal variation, or habitat preference. Likewise, Öztürk [37] reported *An. maculipennis* s.s. as the only representative of the *An. maculipennis* complex in the Central and Eastern Black Sea regions. Together with previous studies, the present findings suggest that *An. maculipennis* s.s. is a dominant and widely distributed member of the complex across diverse ecological regions of Türkiye, likely reflecting its ecological adaptability.

Entomological studies employ a variety of sampling techniques, broadly categorized into collections of adult mosquitoes (flying or resting) and immature stages (larvae and eggs). The choice of sampling method depends on the study objectives, target species, and environmental conditions of the study area. Attractants such as carbon dioxide, odor cues, hosts, and light are effective for capturing mosquitoes at different gonotrophic stages or those actively seeking hosts, whereas mouth aspirators are particularly useful for collecting certain *Culex* and *Anopheles* species that do not readily respond to traps [38,39]. Larval sampling plays a critical role in control and surveillance studies, as it enables the identification of breeding sites and facilitates the detection of species that are either trap-insensitive or present only for short periods. Similarly, egg traps (ovitrap), which mimic oviposition sites, are effective for sampling species that do not respond to conventional trapping methods. In the present study, only adult mosquito samples were collected using New Jersey light traps and mouth aspirators. Although the

results are consistent with findings from previous studies conducted in the region and nearby areas, some members of the complex may not have been detected due to the sampling methods employed. This limitation highlights the potential for sampling bias and suggests that the inclusion of larval and egg sampling could provide a more comprehensive assessment of species diversity in the study area.

Although indigenous malaria transmission has been successfully controlled in Türkiye and no local cases have been reported since 2013, imported malaria cases continue to occur annually ^[1,40]. Recent reports describing *P. falciparum*, *P. vivax*, and mixed infections indicate that international travel, cross-border movement, and increasing human mobility may contribute to changes in malaria epidemiology and the potential reintroduction of malaria in the country ^[2,3,41,42]. Despite *P. vivax* remaining the predominant malaria parasite in Türkiye, the occurrence of imported cases together with the widespread presence of competent vector populations suggests that transmission risk persists under suitable ecological conditions. Furthermore, the Aras Valley, with its suitable microclimate, has been identified as a region in Türkiye favorable for *P. falciparum* transmission, in addition to the lowland areas of the Marmara, Mediterranean, and Southeastern Anatolia regions ^[10]. Given the location of the study area near international borders, monitoring multiple *Plasmodium* species may be important for assessing the risk of imported infections and potential local transmission in the presence of competent vector populations.

Although no malaria parasites were detected in the sampled mosquitoes, the widespread and sustained presence of competent vectors across Türkiye indicates that the potential for malaria transmission persists. Furthermore, climate-driven environmental changes are expected to alter the distribution and biology of both vectors and parasites, potentially increasing the likelihood of malaria re-emergence under favorable conditions. In addition, pathogen surveillance and detection studies require sufficiently large sample sizes to ensure adequate statistical power and reliable detection of low-prevalence infections. Overall, these findings highlight the need for continuous surveillance and expanded sampling efforts to better assess transmission risk and to support early detection of potential malaria resurgence in the region.

DECLARATIONS

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RESEARCH ARTICLE

Economic Analysis of *Tropilaelaps* spp. Infestation in Honeybees, Risk Commodities and Probabilities for Its Introduction in Türkiye

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Abstract

Honeybee pests represent the most significant expense for beekeepers worldwide. While *Tropilaelaps* spp. has not been detected in Türkiye, its presence in neighboring countries poses a critical biosecurity threat. This study conducts a comprehensive risk assessment using the RiskAMP Monte Carlo simulation engine to evaluate the likelihood of introduction and the subsequent economic impact. The model, based on 1.000 iterations, indicates a mean introduction probability of 0.37 per 1.000 events per day, with risk levels ranging from 0.17 to 0.6. The primary introduction pathways identified are bee plundering and the illegal trade of infected colonies, driven by regional price disparities. Our analysis highlights that Türkiye's Mediterranean and Aegean coasts are particularly vulnerable due to high humidity and year-round brood-rearing cycles, which facilitate the parasite's survival. The simulated economic model projects an average annual loss of \$100 million, encompassing direct colony mortality, a significant reduction in honey and wax production, and increased management labor. Additionally, a fixed cost of \$9 million is estimated for mandatory chemical treatments in high-risk climate zones. These findings emphasize the urgent need for enhanced border biosurveillance and regional emergency response plans to protect Türkiye's 9.2 million honeybee colonies and maintain national honey production stability.

Keywords: Economic analysis, Honeybee, Risk, *Tropilaelaps* spp., Türkiye

INTRODUCTION

In recent years, the alarming decline of honeybee populations has raised significant concerns within academic and agricultural communities, particularly with the economic implications of these losses. Various stressors, including pests such as *Tropilaelaps* mites, have been identified as critical threats to bee health and survival, causing widespread damage across the beekeeping industry. *Tropilaelaps*, a genus of parasitic mites, has emerged as one of the more insidious, capable of inflicting considerable harm on honeybee colonies and compromising their productivity. This infestation exacerbates the challenges beekeepers already face, who are grappling with pressures from disease, pesticide use, and habitat loss ^[1,2].

The literature on *Tropilaelaps* infestations in honeybees underscores significant themes related to the ecological impact, economic importance, and management strategies associated with these pests. Many studies highlight the

detrimental effects of *Tropilaelaps* on honeybee health, demonstrating that infestations can lead to population declines and compromised foraging abilities, ultimately affecting pollination services ^[3,4]. This ecological disruption has been linked to broader implications for agricultural productivity, particularly in crops that rely heavily on honeybee pollination, underscoring the economic stakes. As a result, it causes rapid pupal deaths and prepares the environment for secondary bacterial and viral factors ^[5].

Tropilaelaps spp., specifically *T. clareae*, was first identified on honeybees in the Philippines in 1961. Seven years later, these mites spread to honeybee populations in Vietnam, India, and Afghanistan ^[6]. Today, they (*T. clareae*, *T. mercedesae*, or *T. koenigerum*) have been detected in 15 countries across Asia, and cases have also been reported in Kenya and Russia ^[1,7]. Although their range is confined to the Asian continent, they pose a significant threat to global beekeeping. Following observations in Iran ^[8], the Turkish Ministry of Agriculture and Forestry has classified the



mite as a notifiable animal disease in Türkiye. However, to date, there is no evidence of its presence in the country ^[9].

Investigations into *Tropilaelaps* infestations in honeybees unveil a multifaceted canvas of theoretical perspectives that underscore their economic implications. As several studies have shown, the financial impact of these infestations' centres on reduced honey production and increased management costs, which can strain apiarists' finances significantly ^[10]. Theoretical frameworks of ecological interactions suggest that *Tropilaelaps* competes with other parasites, exacerbating vulnerabilities in honeybee populations and cascading effects on pollination services. This interaction is crucial in understanding the broader ecological consequences, including potential declines in agricultural yields that rely on these pollinators ^[11].

This study aimed to assess the introduction of *Tropilaelaps* spp. infestations in Türkiye through the lens of economic modelling. An advanced simulation engine was employed to calculate the direct financial losses incurred by beekeepers, as well as the additional costs associated with insecticide applications. The analysis seeks to provide a comprehensive understanding of the economic impact of these infestations, highlighting both immediate losses and long-term financial implications for the apicultural industry in Türkiye.

MATERIAL AND METHODS

Ethical Statement

The study does not require ethical committee approval.

Data Analyses

A comprehensive spreadsheet was used to evaluate the economic losses utilising the RiskAMP add-in ^[11], a sophisticated Monte Carlo simulation engine that seamlessly integrates with Microsoft Excel. This spreadsheet meticulously incorporates a probability distribution for each entry method, drawing from the identified pathways, with a particular focus on imported commodities ^[12,13].

The simulation leverages a robust computational algorithm that uses repeated random sampling to generate a range of numerical results within the user-defined parameters. This innovative approach not only captures the inherent biological variability but also reflects the diverse spectrum of health-related events. By employing advanced techniques, it is possible to accurately predict future outcomes based on existing risks, providing valuable insights applicable to similar studies ^[14,15].

The parameters and values obtained were input into the simulation engine program to assess risk commodities and their associated probabilities. The selection of model parameters was guided by the most recent official

data (2024) from the Turkish Ministry of Agriculture and Forestry to ensure local relevance ^[16] (*Table 1*). For economic variables such as honey yield, which ranges from 6 to 55 kg per hive, PERT (Program Evaluation and Review Technique) and triangular distributions were used to represent uncertainty while focusing on the most likely outcomes (*Table 2*). This approach was chosen because reliable minimum, maximum, and mode (mean) values are available for these parameters, rather than precise variances. To mitigate the limitations of official data on specific trade volumes, the model incorporates triangular and PERT distributions derived from historical pest emergence patterns and expert biological assessments. By simulating 1.000 iterations, the inherent uncertainty in these parameters is quantified, yielding a confidence interval of \$80 million to \$120 million for direct losses.

Probability distributions for introduction pathways, such as bee plundering and illegal trade, were derived from a synthesis of epidemiological literature and reported infestation statuses in neighboring countries. These distributions account for biological variability and the inherent uncertainties involved in transboundary pest movements, providing a solid foundation for the Monte Carlo simulations.

The level of risk is heavily influenced by the quantities of imports, particularly their country of origin, specifically if they come from infested countries. However, the absence of official data regarding these quantities and sources makes it difficult to accurately assess the risk. Consequently, the potential for disease introduction through imports from infested countries has been evaluated and is now considered an additional risk factor ^[9,12]. To identify the most influential risk factors and interpret the findings, a sensitivity analysis was also conducted.

Table 1. Input parameters used in the economic risk model for Türkiye's beekeeping sector (2024). Data include production averages, unit prices, and professional beekeeper statistics sourced from the Ministry of Agriculture and Forestry

Parameter	Value (2024)
Unit price of a colony	\$70
Average honey production per hive	10.6 kg
Minimum honey production per hive	6 kg
Maximum honey production per hive	55 kg
The average unit price of 1 kg of honey	\$8.5
The average unit price of 1 kg of wax	\$6
No. Professional beekeepers	100.400
Months/year utilized for professional beekeeping	9
Average monthly minimum salary	\$555
Average total colonies in Türkiye	9.2 million

Table 2. Description of potential *Tropilaelaps* spp. introduction pathways into Türkiye, including biological cycles, associated risks, and probability distributions (Min, Mean, Max) used for the Monte Carlo simulation

Commodity/Pathway	Biology Cycle	Description of Risk	Current Practices in Türkiye	Probability
Bee plundering	Adult	Weak colonies in the neighbouring country are plundered by Turkish colonies, which then become infested.	There is a significant population of honey bee colonies in the potential risk area. Areas within 5 km of the border are particularly risky	Worst-case scenario is being considered; therefore, medium to high probability is adopted Min: 0.0001 Mean: 0.0005 Max: 0.001
The illegal introduction of infested colonies and/or other honey bee products from neighbouring country(s)	Eggs, larvae, adult	If a colony is heavily infested with <i>Tropilaelaps</i> , it will die out fast. However, less infested colonies do not die out and remain viable, allowing them to be sold	Although legal import is forbidden, illegal trade involving honey bee colonies and products is possible, especially across borders. Currently, the price of honey in Iran is two times lower than in its neighbours	
Natural spread by flies of bees	Adult	Infested bee, by error, enters a Turkish hive. The new hive must have brood cells because it cannot feed on adult bees; it can only feed on brood	There is a significant population of honey bee colonies in the neighbouring area. Areas within 5 km of the border are particularly risky	Medium Probability Min: 0.00001 Mean: 0.00005 Max: 0.0001
Indirect contact on the same pasture	Adult	<i>Tropilaelaps</i> from the infested bees can remain on a flower and survive for 2 – 3 days, but it is considered infestive only for a few hours. The new hive must have brood cells because it cannot feed on adult bees; it can only feed on brood	There is a significant population of honey bee colonies in the neighbouring area. Areas within 5 km of the border are particularly risky	Medium Min: 0.00001 Mean: 0.00005 Max: 0.0001
Feral bees		The same principles apply to hive bees	The role and influence are not fully clear and must be clarified through research	Medium Min: 0.00001 Mean: 0.00005 Max: 0.0001
Queen bees	Adult	Infested bees or queen bees, together with attendants, will be introduced into the Turkish colony and infestation is inevitable	Minimal quantities are legally imported - no exact figures on imports from the Ministry of Agriculture and Forestry of Türkiye	Very low Min: 0.00001 Mean: 0.00005 Max: 0.0001
Import of comb honey or honey in drums	Eggs, larvae, adult	Comb honey contains brood cells containing eggs, nymphs, larvae and adults. Such imported honey needs to be exposed or introduced into bee colonies	No or minimal quantities are legally imported - no exact figures on imports from the Ministry of Agriculture and Forestry of Türkiye	Very low Min: 0.00001 Mean: 0.00005 Max: 0.0001
The legal import of colonies	Eggs, larvae, adult	Colonies present a significant risk for introducing all mite stages	No or minimal quantities are legally imported - no exact figures on imports from the Ministry of Agriculture and Forestry of Türkiye	Very low Min: 0.00001 Mean: 0.00005 Max: 0.0001

The criteria considered regarding direct and indirect losses from the infestation include: i) loss of bee colonies, honey, and hive frames within the affected colonies; and ii) damage to equipment. The costs associated with disease control are: i) time and labour spent detecting and treating the disease, ii) treatment materials and consumables, and iii) additional expenses for disease control. Possible routes of pest invasion in Türkiye include migratory beekeeping, queen bee importations, and bee plundering ^[9].

RESULTS

The assessment of the risk associated with introducing *Tropilaelaps* spp. through the legal importation of bees and apiculture products highlights a generally low concern. Turkish national legislation effectively restricts the import of bees and honey products from countries

with reported instances of *Tropilaelaps* spp., as specified in the International Veterinary Health Certificate. While the disease may not be detected in exporting countries until it has spread significantly, this situation reduces the likelihood of introduction. Additionally, the possibility of illegal imports, although minimal, was considered during the risk assessment, enabling a comprehensive understanding of the overall risk landscape (Fig. 1).

The data presented in Table 2, along with the assumptions and outcomes of the risk assessment process, suggest that if an infestation were introduced in Türkiye, it could affect any region of the country, as the importation of these commodities is not restricted to specific areas. Therefore, a more thorough investigation is necessary to assess the likelihood of these assumptions and identify preventive measures.

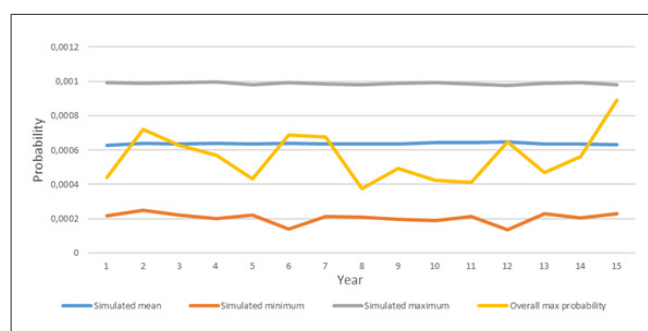


Fig 1. Simulated introduction probability of *Tropilaelaps* spp. in Türkiye over a 15-year projection. The graph displays the results of 1.000 Monte Carlo iterations, showing the simulated mean, minimum, and maximum probabilities alongside the overall maximum probability trend

The simulation indicated that the probability of introducing *Tropilaelaps* spp. in Türkiye ranged from 0.17 per 1.000 events per day (equivalent to 1.7 occurrences every 5500 days or 15 years) to 0.6 per 1.000 events per import (equivalent to 6 occurrences every 5500 days or 15 years). The results showed that bee plundering (11 out of 15 scenarios) and illegal introduction of infected colonies from infected neighbouring countries (4 out of 15 scenarios) had the highest likelihood of causing the infestation. After 1,000 iterations of the constructed model, the mean probability of introducing *Tropilaelaps* spp. was found to be 0.37 per 1.000 events per day (or 3.7 occurrences every 5500 days or 15 years) (Table 3). The temporal shifts in dominant introduction pathways (Table 3) reflect simulated variations in environmental conditions (affecting bee plundering) and regional economic disparities (influencing illegal trade intensity) over the projected 15-year period.

The sensitivity analysis results show that the total economic loss is most dependent on variations in honey yield (6-55 kg/hive) and the honey unit price (\$8.5), while the risk of infestation is most sensitive to the probability of 'bee plundering' (Table 4).

According to the Monte Carlo simulation, after 1.000 simulations, the average assessment of direct losses resulting from the introduction of *Tropilaelaps* spp. in Türkiye is estimated at 100 million US dollars. The assessed losses range from a minimum of 80 million US dollars to a maximum of 120 million US dollars. Calculations include losses of honey, beeswax, and hive frames, as well as bee

Table 3. Simulated 15-year projection of the most dominant introduction pathways for *Tropilaelaps* spp. in Türkiye, based on annual maximum probability outcomes from the Monte Carlo procedure

Year	Route of Introduction	Probability of Introduction (per 1.000)
1	Bee plundering	0.393186284
2	Illegal introduction of infected colonies from infected neighbouring countries	0.603421995
3	Bee plundering	0.267972981
4	Bee plundering	0.249674736
5	Illegal introduction of infected colonies from infected neighbouring countries	0.394877279
6	Bee plundering	0.368415547
7	Illegal introduction of infected colonies from infected neighbouring countries	0.502672639
8	Illegal introduction of infected colonies from infected neighbouring countries	0.394422799
9	Illegal introduction of infected colonies from infected neighbouring countries	0.268425002
10	Bee plundering	0.380063201
11	Bee plundering	0.408641552
12	Bee plundering	0.418406534
13	Bee plundering	0.210367763
14	Illegal introduction of infected colonies from infected neighbouring countries	0.171054827
15	Bee plundering	0.346314887

Probabilities are expressed per 1.000 events. The annual shift in the 'Route of Introduction' reflects the stochastic sampling of environmental and socio-economic variables within the 1.000 iterations of the model

colony mortality and equipment damage. Time spent on detection and treatment, consumables, and additional inspection expenses are included in the cost items. Only professional beekeepers are considered in labor loss calculations; hobby beekeepers are excluded from this item. In line with the literature, the assumption that productivity could decrease by 50% in heavily infected colonies was used to determine the upper limits of the model (Table 5).

Since 80% of the apiaries are located in Türkiye's warmer regions, an additional \$9 million in insecticide costs will be required. The excessive use of these insecticides is expected to worsen the already unfavourable conditions

Table 4. Results of the sensitivity analysis applied to the parameters and data used

Parameter	Interval of Change	Impact on Model Output (Sensitivity)
Honey yield	6 - 55 kg/hive	Very High: Specifies the range of total loss (\$80-\$120M)
Bee plundering	0.0001 - 0.001	High: Indicates the frequency with which the risk of invasion occurs
Honey unit price	\$8.5/kg	Medium: Directly scales the monetary value of the loss
Pest control cost	Fixed rate (80% region)	Low: Adds a linear and constant load to the result

Table 5. Detailed breakdown of economic losses and values used in the simulation

Economic Loss Category	Unit Value (2024) ^[16]	Calculation Method	Computational Logic	Estimated Annual Loss
Colony loss	\$70/Hive	15% colony collapse rate in infected individuals	The cost of replacing colonies that died out as a result of invasion	~ \$30 Million
Product loss	Average honey yield is 10.6 kg; honey price is \$8.50; beeswax price is \$6	40% yield reduction in surviving colonies.	Direct Product Loss	~ \$50 Million
Treatment and control	\$2.5/Hive (Drug). 80% of apiaries in Türkiye are in risky areas	2 extra treatments per year + equipment	A fixed \$9 million was added to the simulation as an additional pest control cost	~ \$11 Million
Labor loss	100,400 professional beekeepers; Minimum monthly wage: \$555	Overtime for disease management (100k beekeepers)	Additional working hours spent on disease diagnosis and further treatment processes	~ \$9 Million
TOTAL		Monte Carlo Simulation Average		~ \$100 Million

related to insecticide presence in honey. Labour loss has been calculated only for professional beekeepers, as hobby beekeepers typically engage in other professions and dedicate minimal time to beekeeping, averaging around 20 days per year.

DISCUSSION

The significance of mitigating pest infestations in honeybee populations has garnered increasing attention, particularly amid rising concerns regarding the ecological and economic roles of these pollinators. The findings from this research indicate that *Tropilaelaps* infestations lead to substantial declines in honeybee productivity, affecting both honey production and pollination efficiency. Specifically, it was identified that colonies with severe infestations could experience a productivity decrease of up to 50% compared to healthy colonies ^[17]. These findings align with previous studies that highlight the economic implications of pest infestations, including *Varroa destructor*, which have led to significant colony losses and financial burdens for beekeepers. Furthermore, the correlation found between infestation severity and various colony health metrics, such as brood viability and adult bee vitality, supports earlier research that has established a direct link between pest pressures and bee health outcomes ^[10,18].

The economic ramifications of *Tropilaelaps* infestations have been approached using quantitative and qualitative methodologies. Quantitative analyses often incorporate economic models to estimate financial losses resulting from reduced honey production and pollination services, underscoring the urgent need for effective pest management strategies ^[19,20]. Conversely, qualitative methodologies, such as interviews with beekeeping experts, have shed light on the social dimensions of infestation management, highlighting the reliance on

traditional knowledge and the evolution of practices over time ^[21]. Overall, the literature reveals a pressing need for continued research employing multiple methodological approaches to address the multifaceted challenges posed by *Tropilaelaps* on honeybee populations and the broader agricultural economy.

Migratory beekeeping practices in Türkiye are diverse, with professional beekeepers travelling an average of 2,000 kilometres annually. Consequently, the introduction of the *Tropilaelaps* mite could pose a serious challenge. With 9.2 million colonies and 85,000 professional beekeepers (those with 50 or more hives) in Türkiye ^[16], there is a significant risk of infestation that threatens sustainable beekeeping.

Risk assessment studies typically focus on meteorological variables or the biology and migratory patterns of beekeeping ^[22,23]. However, there has been no research on the simulated economic risk assessment of *Tropilaelaps* spp., whether in infested or uninfested countries. Therefore, the assessment is the first study conducted in a country free of infestation.

In the absence of local infestation data, the model's outcomes were cross-referenced with existing literature on honeybee mite impacts. The estimated \$100 million loss represents approximately 12-15% of the total annual value of honey production in Türkiye (based on 9.2 million colonies and an average honey price of \$8.5/kg). This ratio is consistent with reported productivity declines of up to 50% in heavily infested colonies elsewhere. The sensitivity of the model to "bee plundering" as the primary introduction route (73% of scenarios) also reflects the biological reality of *Tropilaelaps* movement observed in neighboring regions like Iran and Georgia.

Bee plundering behavior generally increases during dry periods when nectar flow decreases. The "bee plundering"

dominant years in Table 3 represent “famine years” when the parasite spread naturally (from infected colonies in neighboring countries to Turkish colonies). In Türkiye, during years with high winter losses, beekeepers increase their demand for new colonies and queen bees. This situation drives up prices in the local market, encouraging the import of cheaper but uncontrolled bees (illegal trade) from neighboring countries (Iran, Georgia, etc.). The peaks in Table 3 model these types of “market pressure” scenarios.

Although honey prices in Türkiye (\$8.5/kg) are lower than local prices in Georgia (average \$15/kg)^[24], they are approximately 50% higher than prices in Iran (average \$4.5/kg)^[25]. This price difference is a key economic driver encouraging the illegal import of bees and bee products.

The literature^[2,5] consistently states that intra-hive temperatures of 32-35°C and high humidity are necessary for optimal development of *Tropilaelaps* spp. Türkiye's southern coastal region (Antalya, Mersin, Adana) and the Aegean region (Muğla, Aydın) exhibit characteristics similar to the humid and warm climate of Southeast Asia, the parasite's native habitat. The fact that bees in the Mediterranean and Aegean regions do not form winter clusters and continue brood rearing creates an “excellent” environment for the parasite to establish itself in Türkiye. In Central and Eastern Anatolia, however, the cessation of winter clusters and brood rearing would break the parasite's life cycle. In migratory beekeeping in Türkiye, most hives are relocated to warmer regions such as Muğla and Antalya during winter. This practice poses a risk of “human-caused spread” that exceeds climatic barriers.

To prevent the direct spread of infestations, beekeeping should be prohibited in areas within 10 km of the borders of infested countries. A thorough investigation is needed to assess the likelihood of these assumptions and to develop effective prevention measures. An early warning system should be established near the border with Iran to alert authorities about any potential introduction of the infestation. Additionally, it is essential to collaborate with veterinary authorities to receive updated information on the presence and distribution of the disease in Iran, Azerbaijan, and Georgia^[3,8,26].

In conclusion, this study conducted a comprehensive analysis of the significant economic impacts of *Tropilaelaps* mites in Türkiye, highlighting the critical risks of contamination associated with their emergence. Using advanced simulation models, detailed insights into the financial burdens imposed by this undetected honeybee parasite were obtained. The compiled data highlights high costs and serves as a resource for developing a comprehensive financial model that can be adapted for other uninfested countries. This research emphasises

the urgent need for vigilance and proactive measures in protecting honeybee populations worldwide.

DECLARATIONS

Availability of Data and Materials: All data and materials are kept in the Department of Parasitology laboratory at the Veterinary Faculty, Bursa Uludag University.

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Authors' Contributions: LA conceived and designed the analysis, and interpreted the outputs; OG, AOG, İAÖ, and DT contributed the data and analysis tools; AOG and OG finalized the work presentation.

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RESEARCH ARTICLE

Re-freezing of Cryopreserved Equine Semen: Effect of Glycerol Concentration, Cholesterol, and α -Tocopherol on Sperm Quality

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Abstract

The present study evaluated the effect of glycerol concentration, treatment with cholesterol and α -tocopherol loaded into methyl- β -cyclodextrin, and sperm cell concentration on the quality of equine spermatozoa after two cycles of cryopreservation. Two experiments were conducted. The first trial utilized 18 ejaculates collected from six stallions. Each ejaculate was evaluated initially using computer assisted sperm analysis (CASA) and flow cytometry (FCM), and spermatozoa were subsequently frozen in a commercial diluent (containing 2.5% glycerol: control) or in one of four experimental diluents (each supplemented with 1%, 2%, 3% or 4% glycerol). Frozen samples were thawed and evaluated again prior to a second freeze-thaw cycle. The glycerol concentration that produced the best result was further investigated in experiment 2 where spermatozoa previously frozen in 2.5% glycerol were thawed and refrozen in one of three sperm cell concentrations (1, 5, 05 10 x10⁶ spermatozoa/mL) treated or not treated with cholesterol and α -tocopherol incorporated into methyl- β -cyclodextrin. Semen analysis (CASA and FCM) was repeated after the second freezing and thawing. Results revealed that the 2.5% concentration of glycerol was optimal for refreezing equine spermatozoa. Centrifugation (900xg for 10 minutes) of the equine semen before refreezing resulted in a sperm loss rate of 12% but was associated with good viability and acrosomal integrity. Treatment of semen with cholesterol and α -tocopherol loaded into methyl- β -cyclodextrin before refreezing improved ($P < 0.05$) the post-thaw quality of spermatozoa (motility, membrane and acrosome integrity, and oxidative status) refrozen at 1×10^6 spermatozoa/mL, thus allowing production of 100 to 300 ICSI straws from a standard 0.5 mL equine artificial insemination straw.

Keywords: Cryopreservation, Sperm, Glycerol, Methyl-B-cyclodextrin, Stallion, Vitamin E

INTRODUCTION

In equines, conventional *in vitro* fertilization (IVF) has been less successful than in many other species. This lower success rate constrains embryo production and has led researchers and practitioners to utilize an alternative fertilization technique called intra-cytoplasmic sperm injection (ICSI) [1]. To use ICSI, mares must undergo egg retrieval (also known as ovum pick-up [OPU]) to harvest immature oocytes that will be matured *in vitro* prior to fertilization via ICSI. This *in vitro* embryo production procedure has proven very effective and has become widely used in mares. However, with the increased use of ICSI, another issue has arisen - namely the cryopreservation of a small number of male gametes needed for ICSI [2].

Unlike artificial insemination (AI) where the inseminate dose is approximately 400 million spermatozoa/mL [3], ICSI requires only a single spermatozoon recruited from a small quantity of cells. Thawing an entire AI straw for the minimal needs of ICSI would be a waste of semen, particularly for the limited supplies of semen available globally from stallions of very high genetic and sporting value [4]. Thus, the ability to thaw stallion spermatozoa cryopreserved in straws at a concentration between 100-300 million spermatozoa/mL and subsequently refreeze them in so-called ICSI straws at a much lower concentration (1-10 million spermatozoa/mL) would facilitate greater gains in embryo production [5,6].

In horses, refreezing of stallion semen was first reported in 2006, although those procedures resulted in immobile sperm. After ICSI with immotile sperm, embryonic



development to the blastocyst stage was achieved, but with a low conception rate compared with that obtained using motile spermatozoa resulting from a single freeze-thaw cycle [7]. Several studies were performed after that initial report, but to date no one has succeeded in obtaining satisfactory quality (motility and integrity) of spermatozoa after refreezing. In fact, the re-freezing process exposes spermatozoa to damage - causing a deterioration of their motility, integrity and metabolism. This damage leads to a considerable reduction in fertilizing capacity, particularly for the equine spermatozoon which is sensitive to cold temperatures due to a low cholesterol:phospholipid membrane ratio compared with other species [5,8]. Lesions following freezing are due to osmotic stress acting in conjunction with oxidative stress through free radical elimination [9]. Therefore, treatment of the spermatozoa with cholesterol and α -tocopherol before re-freezing could increase the cryo-resistance of the equine sperm cell by stabilizing the plasma membrane and preventing the accumulation of free radicals (oxidative stress).

Cryoprotective agents (e.g., glycerol) protect spermatozoa during cryopreservation by minimizing their exposure to osmotic stress, stabilizing the plasma membrane, and reducing the deleterious effect of free radicals [10,11]. However, glycerol also has a dose-dependent toxic effect on spermatozoa, particularly during prolonged and/or repeated exposure during refreezing. For this reason, glycerol concentration in the freezing diluent should be optimized [12,13].

The present study evaluated the effect of glycerol concentration, final sperm cell concentration, and treatment with cholesterol and α -tocopherol loaded into methyl- β -cyclodextrin before re-freezing on the quality (motility, viability-integrity, oxidative stress) of equine sperm through two cycles of cryopreservation.

MATERIAL AND METHODS

Ethical Approval

All animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the course of the work. All experiments were conducted according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Study Area

The experiments were performed in the laboratories at BIOMERA Platform, Blida University 1 (Blida, Algeria).

Animals

Six clinically healthy purebred Arabian stallions of proven fertility were used for semen collection (fertility

was assessed through results of AI with fresh semen). Stallions were chosen from one of two different farms (Haras Hocine El Mansour Mostaganem and the Sahel Hadjout Equestrian Farm), and they ranged from four to nine years of age. Although semen analysis is performed routinely on all stallions housed on those two farms, the six stallions were chosen at random from among those with satisfactory semen characteristics in order to avoid any potential bias/sampling effect.

Semen Collection

During the week preceding their use for experiments, stallions underwent semen collection three times to empty their extra-gonadal reserve of spermatozoa with the aim of ensuring a constant quality of semen during the experiments. During the experiments, semen was collected at least twice per week from each stallion. Semen collections from the same stallion were not carried out on two consecutive days to maintain an adequate sperm cell concentration. Stallions were, therefore, collected alternately in groups of two. Semen collection was done using a closed artificial vagina (Colorado type) at the beginning of the afternoon on a phantom in the presence of a mare in heat, or directly on the mare for stallions not trained to a phantom.

Once collected, semen was immediately filtered into a graduated container using filter paper or sterile double gauze to remove impurities and the "gel" which contains seminal fluid that is highly toxic for spermatozoa during cryopreservation. The artificial vagina and all materials that came in contact with semen were preheated at 37°C to avoid any possible thermal shock. The volume of the filtered ejaculate was measured, and the ejaculate was then aliquoted into four 50-mL tubes pre-warmed to 37°C.

Initial Assessment of Semen

Initial quality of the ejaculate was assessed using three criteria: 1) macroscopic evaluation of the physicochemical parameters of the ejaculate (volume, color, appearance, smell); 2) assessment of the sperm cell concentration using an "Improved Neubauer" chamber after a 1:100 dilution (10 μ L of semen and 990 μ L of 4% NaCl solution); 3) assessment of motility by placing a drop (10 μ L) between a slide and a coverslip and visually examining it under a microscope by looking at several fields and making an average estimation (expressed as a percentage or using a corresponding grid from 0 to 5) of the spermatozoa with slow and fast rectilinear movement (Haras Nationaux, 2009).

Preparation of Solutions Loaded into Methyl- β -Cyclodextrin

Cholesterol and α -tocopherol were incorporated into methyl- β -cyclodextrin (CLC: cholesterol loaded methyl-

β -cyclodextrin; TLC: α -tocopherol loaded methyl- β -cyclodextrin, respectively) according to previously reported methods ^[15,16]. Two solutions were prepared for treatments to be applied to spermatozoa prior to re-freezing at the following concentrations: 10.86 mg/mL (CLC) and 0.92 mg/mL (TLC). A third solution (CLC and TLC combined) was prepared by mixing the CLC and TLC solutions in equal proportion (v/v). All treatment solutions were filtered using a syringe filter and subsequently stored at +4°C until use. The CLC treatment dose was 5 mg/100x10⁶ spermatozoa, and the TLC treatment dose was 0.1 mM/100x10⁶ spermatozoa. The dose of the combination treatment (CLC+TLC) consisted of a half dose of each of the two treatments.

Semen Dilution and Temperature Management

The diluents used for these experiments was INRA96® (IMV, L'Aigle, France). To avoid any potential unintentional atmospheric alteration of the diluent after opening the 200 mL vial, the INRA96® was aliquoted into 5, 10, 20, and 50 mL quantities and frozen at -18°C (for ≥ 24 h). On each day of semen collection, the needed volume of INRA96® was thawed at room temperature and then warmed to 37°C in a water bath. The warmed INRA96® was slowly added to the semen for a 1:3 dilution (i.e., 3 volumes of INRA96® per 1 volume of semen).

Tubes containing the diluted sperm were placed in a water bath at 22°C for 10 min to initiate the cooling process. For further slow cooling, tubes with diluted semen were placed in plastic containers filled with 22°C water for subsequent placement into a 4°C refrigerator for 30 min. This procedure enabled cooling of the semen to occur slowly, avoiding temperature shock to the spermatozoa. After 30 min, the semen doses were packaged in insulated Equitainers and transported at 4°C to the BIOMERA platform in Blida. Average transport time was 3 h.

Upon arrival at BIOMERA, semen was layered on top of 2 mL of centrifugation cushion designed for equine spermatozoa (MaxiFreeze, IMV, L'Aigle, France) in a 10mL tube. Sperm cells were concentrated by centrifugation (SIGMA 3-30KS centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 900 x g for 10 min. This centrifugation protocol was expected to result in loss of approximately 15% of spermatozoa ^[17].

After removing the supernatant (first diluents D1 + seminal plasma), the sperm pellet was reconstituted using the freezing diluent (2nd diluent D2). We calculate the theoretical volume of D2 based on the sperm concentration measured before centrifugation (C1) to obtain a final concentration of 100x10⁶/mL (C2) according to the relation ($C1 \cdot V1 = C2 \cdot V2$). Half of the theoretical volume of D2 ($\frac{1}{2}$ Vol of D2) was added per tube, and the spermatozoa were resuspended via gentle

pipetting by hand, then the final sperm concentration of the reconstituted sample was determined as described previously. The volume of D2 remaining to be added will be calculated as described previously based on the concentration measured after centrifugation in order to correct the volume for losses due to centrifugation.

Straw Filling, Freezing, and Thawing

After 120 min of equilibration time at 4°C, straws individually labeled using a printer (Domino A420i, Domino Printing, Cambridge, UK) were filled and sealed with a filling machine (MRS I DualV2, IMV, L'Aigle, France). The straws were spread on a freezing rack, and the rack was placed in an automatic freezer (MicroDigitcool, IMV, L'Aigle, France; Curve: Start at 4°C; - 60°C/min up to -100°C; -30°C/min to -140°C). At the end of the freezing cycle, straws were immersed directly in liquid nitrogen (LN2). Any straw that floated in LN2 due to insufficient filling was eliminated.

After 72 h of storage, straws were placed into a water bath at 37°C for 30 seconds for thawing.

Semen Evaluation

Motility Analysis by Computer-Assisted Sperm Analysis (CASA): Pre- and post-thaw sperm motility was determined via computer-assisted sperm analysis (CASA). To prepare spermatozoa for analysis, semen was diluted to a concentration of 25 million spermatozoa/mL using a commercial buffer solution (EasyBuffer B, IMV, L'Aigle, France) and subsequently incubated in water bath (MEMMERT incubator, Memmert, Büchenbach, Germany) at 37°C for 10 min. Spermatozoa were loaded into a 20 μ m deep counting chamber (Leja®, LEJA Product BV, Netherlands) and analyzed with a Hamilton Thorne motility analyzer system (IVOS® II, Beverly, Massachusetts, USA) in accordance with a previously described technique ^[18]. The IVOS®II technical configurations shown in [Table 1](#). The two parameters evaluated were percentages of motility (MOT) and progressive motility (PROG).

Viability, Acrosomal Integrity, and Sperm Oxidative Status Assessment by Flow Cytometry (FCM): Sperm viability, acrosomal integrity, and oxidative status were assessed via flow cytometry (Millipore GuavaEasyCyte HT Plus, Merck KgaA, Darmstadt, Germany) using ready-to-use rapid kits in the form of microplates containing fluorochromes in their wells. After several steps of sperm dilution and preparation, sperm were added to the fluorochrome-containing wells, incubated, and loaded directly into the flow cytometer for analysis. A sample of fluorescently labeled cells is aspirated into a uniquely proportioned microcapillary flow cell. A diode laser excites the cells and each cell emits signals

Table 1. Parameters for computer-assisted sperm analysis of equine spermatozoa using a Hamilton Thorne IVOS II CASA analyzer

Parameter	Value
Frame acquired	30
Frame rate (Hz)	60
Minimum contrast	60
Minimum cell size (pixels)	6
Minimum static contrast	25
Straightness cut-off (STR, %)	75
Average path velocity cut-off PM (VAP, $\mu\text{m/s}$)	50
VAP cut-off static cells (VAP, $\mu\text{m/s}$)	20
Cell intensity	100
Static head size	0.55-2.04
Static head intensity	0.45-1.70
Static elongation	11-99

that are individually detected by photomultipliers and a photodiode. The excitation laser provides up to five simultaneous detection parameters, including three fluorescent colors plus forward and side scatter for size and complexity determination. Software modules show all relevant data and results immediately.

The percentage of living spermatozoa with an intact acrosome was determined with the kit "Fluorochromes: FITC/PNA/PI" (VIAB-ACRO). A new combination of three stains was developed by IMV. Two of the stains monitor the integrity of the acrosome and of the membrane, simultaneously. The third fluorochrome is sperm specific and thus allows to remove debris from the analysis.

The oxidation status was determined by measuring the level of three intracellular Reactive Oxygen Species (hydrogen peroxide " H_2O_2 ", hypochlorous acid " HOCl ", and peroxynitrite " ONOO^- ") of the sperm through the use of flow cytometry, by combining cell and viability markers, as described previously in the principle of this technique. Physical levels of ROS are required for normal sperm functions. However, when in excess, ROS can be harmful. Oxidative stress occurs in spermatozoa when intra and extra cellular levels of ROS exceed the available antioxidant capacity.

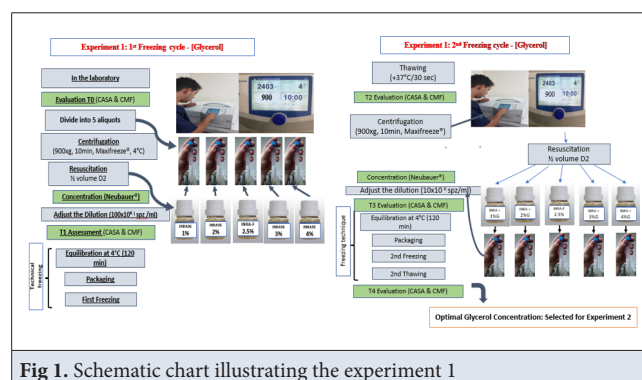
Experimental Design

Experiment 1- Evaluation of Glycerol Concentration and Effect of Centrifugation: Ejaculates from stallions were collected and processed as described earlier. After initial assessments (T0), ejaculates were split in half and allocated to the control (INRAFreeze®, IMV, L'Aigle, France; contains 2.5% glycerol [G]) or to one of four experimental diluents (INRA96®+1%G, INRA96®+2%G, INRA96®+3%G,

INRA96®+4%G) prepared by supplementing INRA96® with 1%, 2%, 3%, or 4% G, respectively. Spermatozoa were evaluated (CASA & FCM) after centrifugation (T1), equilibrated at 4°C for 120 min, and packaged in 0.5 mL straws before being frozen with an automatic freezer (Curve: Start at 4°C; - 60°C/min up to -100°C; -30°C/min to -140°C) and subsequently stored in LN₂ for three days. Three straws from each group were thawed (+37°C for 30 sec) in five different tubes, evaluated (CASA& FCM; T2), and then centrifuged (900xg for 10 min on cushion) and resuspended in ½ volume of the corresponding diluent. Sperm concentration was assessed after centrifugation and a volume of diluent calculated was added to have a final concentration of 10×10^6 spz/mL in the five tubes. A new evaluation (CASA & FCM) was performed (T3) before spermatozoa were refrozen exactly as described for the first freezing cycle. Three straws/treatment group were thawed and evaluated after the second freezing and thawing event (CASA & FCM; T4).

A priori, it was decided that the G concentration giving the best result would be retained and used as the basis for experiment 2 (Fig. 1).

Experiment 2- Effect of Cholesterol and α -Tocopherol, and Sperm Cell Concentration: Based on results from experiment 1, semen frozen in INRA-Freeze® was used for experiment 2. Six straws (3 mL total volume of semen) were thawed as described in experiment 1 and then analyzed (CASA and FCM) at T0. The thawed ejaculates were centrifuged (900xg for 10 minutes) and resuspended before being aliquoted into one of three tubes at different dilutions (1:10; 1:20; 1:100) corresponding to three final sperm concentrations (10×10^6 spermatozoa/mL; 5×10^6 spermatozoa/mL; 1×10^6 spermatozoa/mL, respectively). Each of the 3 tubes subsequently was aliquoted into two tubes - one containing the combined CLC+TLC treatment and the other serving as an untreated control. The six tubes were equilibrated, and semen was packaged in straws, frozen, stored, and then thawed and analyzed (CASA and FCM) exactly as described in experiment 1 (Fig. 2).

**Fig 1.** Schematic chart illustrating the experiment 1

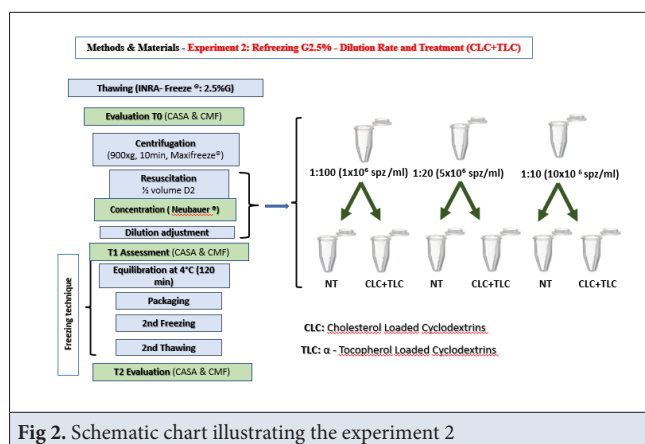


Fig 2. Schematic chart illustrating the experiment 2

Statistical Analysis

The SPSS statistical software was used for analysis of data, including the generation of descriptive statistics (mean, standard deviation, standard error of the mean); testing if response variables were normally distributed; performance of a logarithmic transformation if data were not normally distributed; conduct of an analysis of variance (ANOVA) and mean separation tests (Tukey and Duncan tests; Post hoc). The significance level was set at 5% ($P < 0.05$), and results are presented as Mean $\pm$ SEM (standard error of the mean).

RESULTS

Initial evaluation of the semen immediately after collection showed a mean concentration of $155.5 \pm 4.21 \times 10^6$ spermatozoa/mL and an average mass motility score of 3.5 corresponding to around 70% of spermatozoa with slow and fast rectilinear movement.

Experiment 1- Evaluation of Glycerol Concentration and the Effect of Centrifugation

Sperm parameters were evaluated at five different time points during experiment 1. The initial analysis after 3 h of transport (T0) (Table 2), after the first centrifugation (T1) (Table 3), after the first thawing (T2) (Table 4), before

Table 2. Initial sperm parameters (n=18 ejaculates) after three h of transport (T0- Exp1)

Parameter [‡]	Value*
Concentration ($\times 10^6$ spermatozoa /mL)	155.5 $\pm$ 4.21
VIAB/ACRO (%)	54.0 $\pm$ 2.30
ROS (%)	23.8 $\pm$ 0.73
MOT(%)	78.6 $\pm$ 1.55
PROG (%)	54.6 $\pm$ 2.33

[‡] VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

* Expressed as mean $\pm$ standard error of the mean (SEM)

the second freezing (T3) (Table 5), and after the second thawing (T4) (Table 6).

The comparison of sperm parameters between the first and the second thawing in different cryopreservation solutions showed a decrease ($P < 0.05$) in motility parameters as well as in the percentage of spermatozoa that were alive with intact plasma and acrosomal membranes (Fig. 3). Comparing glycerol concentrations, data revealed that the only difference observed was in MOT; the 2.5% glycerol concentration was better than the 1% and 4% but not different than the 2% and 3%.

For the diluent INRA-Freeze®, the percentage of motile (MOT), progressively motile (PROG), and viable spermatozoa with intact acrosomes dropped considerably after refreezing with values of 51.40 vs. 9.17%; 42.74 vs. 7.63%; 52.84 vs. 21.39%, respectively. Oxidative damage to spermatozoa increased ($P < 0.05$) from 26.39 to 39.83%.

The effect of centrifugation on spermatozoal parameters during the refreezing cycles was also examined. Centrifugation decreased ($P < 0.05$) sperm cell concentration with a loss rate of 12.81% in the second centrifugation (refreezing). For motility (MOT and PROG) and acrosome viability, no differences ($P > 0.05$) were observed due to centrifugation (Fig. 4; Table 5).

Experiment 2- Effect of Cholesterol and α -Tocopherol, and Sperm Cell Concentration

A comparison of sperm parameters between the first and second thawing in each of the three sperm cell concentrations (1×10^6 ; 5×10^6 ; 10×10^6) is presented in Table 7 and Table 8. At the two highest sperm cell concentrations, there was no difference ($P > 0.05$) between concentrations in sperm motility, sperm integrity, or oxidative stress level; however, those sperm parameters were lower ($P < 0.05$) at a concentration of 1×10^6 spermatozoa/mL than at the other two concentrations.

Treatment of spermatozoa with a combination of cholesterol and α -tocopherol loaded into cyclodextrin (CLC+TLC) improved ($P < 0.05$) motility, integrity and oxidative status (Table 9), with the exception of motility (MOT and PROG) in the highest sperm cell concentration (10×10^6 spermatozoa/mL) which was unaffected.

DISCUSSION

Conventional sperm selection techniques used in reproductive biotechnologies rely on centrifugation. Investigations that evaluated the influence of centrifugation on stallion sperm quality revealed conflicting results [19,20]. In the present study, stallion spermatozoa underwent two centrifugations with a standard protocol (900xg for 10 min), as it was deemed essential when preparing

Table 3. Initial sperm parameters (n=18 ejaculates) after the first centrifugation (T1- Exp1)

Parameter [‡]	Diluent [*]				
	INRA-Freeze	INRA96+1% G	INRA96+2% G	INRA96+3% G	INRA96+4% G
Concentration (x10 ⁶ spermatozoa/mL)	132.63±3.49 ^a	130.43±3.45 ^a	127.95±3.44 ^a	132.63±3.49 ^a	132.34±3.38 ^a
VIAB/ACRO (%)	53.40±2.28 ^a	51.87±2.22 ^a	52.84±2.25 ^a	51.23±2.19 ^a	50.29±2.16 ^a
ROS (%)	23.54±0.72 ^a	23.35±0.71 ^a	23.79±0.73 ^a	23.06±0.71 ^a	22.64±0.69 ^a
MOT (%)	78.40±1.49 ^a	75.79±1.45 ^{bc}	76.56±1.44 ^a	73.04±1.38 ^{ab}	68.29±1.33 ^c
PROG (%)	53.64±2.2 ^a	52.61±2.24 ^a	53.13±2.24 ^a	50.69±2.15 ^a	49.21±2.08 ^a

* G denotes glycerol; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)

‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

Table 4. Sperm parameters (n=18 ejaculates) after the first thawing with different levels of glycerol added to diluents (T2-Exp 1)

Parameter [‡]	Diluent [*]				
	INRA Freeze	INRA96+1%G	INRA96+2%G	INRA96+3%G	INRA96+4%G
VIAB/ACRO (%)	52.84±2.25 ^a	45.02±1.43 ^b	51.32±2.23 ^{ab}	50.13±1.43 ^a	44.01±1.43 ^c
ROS (%)	26.90±0.67 ^a	33.78±0.51 ^b	30.88±0.84 ^c	29.10±0.51 ^{ac}	33.21±0.64 ^{cb}
MOT(%)	51.40±1.14 ^a	35.55±0.68 ^b	46.06±0.81 ^a	46.65±0.92 ^a	31.93±0.77 ^c
PROG(%)	42.74±1.68 ^a	30.79±0.73 ^b	41.97±1.10 ^a	40.68±1.34 ^a	29.25±0.90 ^c

* G denotes glycerol; INRAFreeze contains 2.5% G; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)

‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

Table 5. Sperm parameters after the second centrifugation with the different levels of glycerol added to the diluents before the second freezing (T3-Exp 1)

Parameter [‡]	Diluent [*]				
	INRA-Freeze	INRA96+1%	INRA96+2%	INRA96+3%	INRA96+4%
Concentration (x10 ⁶ spermatozoa/mL)	113.13±3.17	111.43±3.12	109.76±3.08	108.25±3.08	106.51±3.05
VIAB/ACR (%)	45.75±2.02	44.44±1.97	45.26±2.00	43.89±1.95	43.08±1.92
ROS (%)	35.85±1.24	36.90±1.28	36.23±1.25	37.36±1.29	38.04±1.32
MOT (%)	50.46±1.11	48.62±1.07	49.53±1.09	47.73±1.05	46.86±1.03
PROG (%)	41.96±1.65	40.44±1.60	41.19±1.63	39.70±1.58	38.98±1.56

* G denotes glycerol; INRAFreeze contains 2.5% G; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)

‡ VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

equine semen for freezing and refreezing [21]. The first centrifugation enabled elimination of the seminal plasma (containing a lot of frost) with known deleterious effects on sperm quality as well as on the centrifugation diluent. The second centrifugation allowed removal of the original freezing medium whose composition had presumably been altered and potentially loaded with toxins; it was replaced with fresh freezing medium for the second cycle of cryopreservation [22]. However, centrifugal force exposed spermatozoa to the risk of mechanical shock, likely altering their quality and increasing risk of cell loss in the supernatant [23]. Thus, we evaluated the motility and integrity parameters, as well as sperm cell concentration, before and after each centrifugation to assess any effect of

centrifugation on semen quality. As the main aim of this study is to split a frozen 0.5 mL straw into as many ICSI subunits of good quality as possible after thawing and refreezing, the benefit of repeated centrifugation before the second freezing seems questionable.

Results of the first experiment showed a decrease in sperm cell concentration in the second centrifugation (refreezing) with a loss rate of 12.81%. This loss rate is lower than the 22% loss rate reported previously using the same centrifugation protocol [23]. With respect to motility (MOT and PROG) and acrosomal integrity, centrifugation had no effect. Although the centrifugation protocol caused a loss of spermatozoa, it preserved motility and sperm integrity satisfactorily. This result is contrary to a previous study

Table 6. Sperm parameters (n=18 ejaculates) after the second thawing with different levels of glycerol added to diluents (T4-Exp 1)

Parameter [‡]	Diluent *				
	INRA-freeze	INRA96 + 1%G	INRA96 +2%G	INRA96 + 3%G	INRA96 +4%G
VIAB/ACRO (%)	21.39±1.02 ^a	18.90±0.77 ^b	21.17±1.01 ^a	20.52±0.99 ^a	17.68±0.63 ^b
ROS (%)	39.83±1.22 ^a	47.57±1.63 ^{bc}	44.50±1.65 ^{cd}	45.88±1.70 ^d	50.44±1.59 ^e
MOT (%)	9.17±0.22 ^a	7.79±0.25 ^b	9.00±0.22 ^a	8.67±0.21 ^{ab}	7.38±0.28 ^{cb}
PROG(%)	7.63±0.32 ^a	6.35±1.18 ^b	7.49±0.31 ^a	7.22±0.31 ^{ab}	6.17±0.17 ^{cb}

* G denotes glycerol; INRAFreeze contains 2.5%G; values are expressed as mean ± standard error of the mean (SEM); means with unlike superscripts within a row are different (P<0.05)
[‡] VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

[24] where various centrifugation protocols (300× g for 5 min, 300× g for 10 min, 1500× g for 5 min, 1500× g for 10 min) impaired motility and increased oxidative damage to cryopreserved stallion spermatozoa, even at a weak force for a short time. More recently, colloidal centrifugation and filtration techniques were compared with conventional simple centrifugation following cryopreservation, and no discernible improvement in semen quality was observed when using these alternate techniques [25]. On the contrary, Gutiérrez-Cepeda et al. [26] found that colloidal centrifugation optimized the efficiency of cryopreservation, as it allowed to increase the number of ejaculates suitable to be frozen, especially when dealing with individuals or breeds for which initially low sperm quality prevents or limits their inclusion in sperm cryopreservation programs.

The loss of spermatozoa in the present investigation, although lower than other studies, may have resulted from the low speed/force used (900xg for 10 min) which in turn did not allow all of the sperm to enter the sperm pellet prior to reconstitution. High speeds/forces of centrifugation (1200, 1800, 2400xg) were associated with lower sperm losses (23.0, 7.4, and 2.1%, respectively) but

also with reduced sperm quality following mechanical shock [23].

The preservation of sperm motility and integrity after centrifugation in our study could be explained by the use of a centrifugation cushion which prevented the percussion shock of the spermatozoa against the wall of the centrifuge tube. This protective effect has been widely reported in the literature [21,22,27]. An additional measure to combat mechanical shock to the sperm was applied in our study; the rotor brake of the centrifuge was deactivated, thus allowing the centrifuge to stop gradually (sudden stoppage increases the mechanical shock inflicted on spermatozoa).

Sperm motility and integrity were compared between the first and the second freezing cycle in the present study, and results showed that equine sperm suffered significantly through this double freezing procedure. Excessive degradation of sperm quality after re-freezing has been reported in the literature [7,28]. Degradation after the second freeze/thaw cycle could be explained by the action of cold temperature and glycerol on cells already weakened by the first cycle of cryopreservation. In a different experiment using INRA82, Sielhorst et al. [29] reported the first study evaluating sperm fertility after three freezing cycles of stallion spermatozoa. These authors found a pregnancy

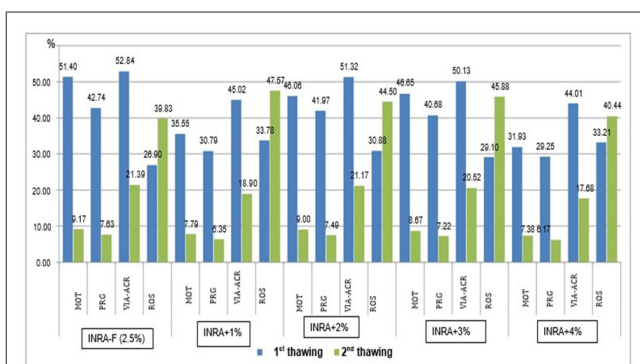


Fig 3. Motility (MOT), progressive motility (PRG), viability with an intact acrosome (VIA-ACRO), and oxidative damage due to reactive oxygen species (ROS) in frozen-thawed equine spermatozoa after the first and the second thawing. Spermatozoa were cryopreserved in diluents containing one of five different levels of glycerol: 2.5% for INRA-Freeze (INRA-F); 1%, 2%, 3%, and 4% for INRA+1, INRA+2, INRA+3, and INRA+4, respectively. All values within a diluent and sperm parameter, all values are different (P<0.05)

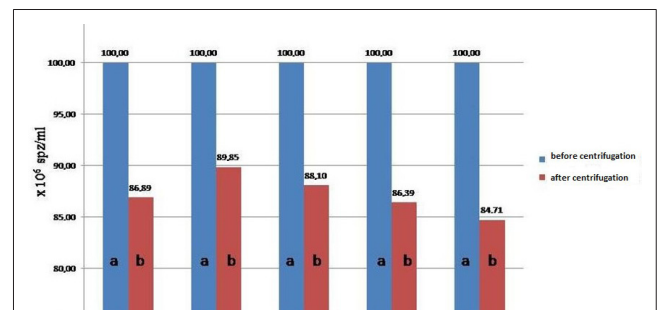


Fig 4. Effect of centrifugation on sperm cell concentration during the second freezing of equine spermatozoa (spz) in different cryopreservation diluents (Exp1). (INRA-F = INRA-Freeze containing 2.5% glycerol, INRA+2 = INRA96® + 2% glycerol, INRA+3 = INRA96® + 3% glycerol, INRA+4 = INRA96® + 4% glycerol. Sperm cell concentrations in each diluent were set equivalent to 100% prior to centrifugation. Means within a diluent with unlike superscripts are different (P<0.05; n=18 ejaculates)

Table 7. Spermatic parameters (n=18 ejaculates) after the first thawing (T0-Exp 2)

Parameter [‡]	1:10 Ejaculate Dilution
VIAB/ACRO (%)	52.39±2.14
ROS (%)	28.62±0.92
MOT (%)	50.39±1.11
PROG (%)	41.91±1.66

[‡]VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

Table 8. Spermatic parameters after the second thawing (T1-Exp 2) in the three dilutions studied (P<0.05; n=18)

Parameter [‡]	Dilution		
	1:100	1:20	1:10
VIAB/ACRO (+) (%)	14.05±1.07 ^a	20.56±0.98 ^b	20.97±1.00 ^b
ROS (%)	43.19±1.62 ^a	28.92±1.83 ^b	27.35±1.61 ^b
MOT(%)	6.07±0.37 ^a	8.99±0.21 ^b	8.81±0.21 ^b
PROG(%)	5.03±0.36 ^a	7.48±0.31 ^b	7.34±0.31 ^b

[‡]VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

rate of 40% with semen frozen once and 10% with double-frozen semen. They revealed after the third freeze/thaw cycle, that the sperm quality decreased to less than 10% motile and membrane-intact cells, which resulted in no pregnancy after conventional AI. A reduction in sperm quality after refrigeration (4°C) is considered normal, as the decreased temperature can induce changes that may have a detrimental impact on semen quality and fertility^[25]. In another study, Leisinger et al.^[28] confirmed that DNA denaturability was not affected by semen packaging method and was only affected by thaw number, increasing at post-thaws 5 and 6.

Fertilization, the most fundamental success criterion to assess after sperm cryopreservation, requires the

functional integrity of the sperm plasma membrane to be maintained throughout the freezing and thawing processes. This functional integrity is correlated with motility^[30]. Cold shock, osmotic pressure change, ice crystal formation, and generation of reactive oxygen species (ROS) can all cause changes in the lipid composition of the cell membrane^[31]. These changes negatively affect sperm motility, viability, mitochondrial membrane function, DNA integrity, and fertilizing ability of spermatozoa^[32].

The appropriate thawing rate can be influenced by many aspects of the cryopreservation procedures, including diluent type, glycerol concentration, and freezing curve^[33]. Glycerol (G) is the most commonly utilized membrane-permeable cryoprotectant which restricts intracellular ice crystal formation^[34]. However, it also exerts a dose-dependent toxic effect on spermatozoa-especially with prolonged and/or repeated exposure to refreezing^[12]. The optimal concentration of G to use in diluents is not standardized yet, and published works revealed contradictory data^[35]. For this reason, we evaluated the effect of five different concentrations of G (1%, 2%, 2.5%, 3% and 4%) in the freezing medium for optimization purposes. Results from experiment 1 revealed that the control medium containing 2.5% G offered better preservation of motility (MOT and PROG) and membrane and acrosomal integrity (VIAB-ACRO) as well as better protection against oxidative stress (ROS) compared with other G concentrations during two freezing cycles. Concentrations of 1% and 4% G gave poorer results than those obtained with 2.5% G for all parameters studied compared. It seems that 1% G is insufficient to effectively protect the spermatozoa from intracellular ice crystal formation and poorly preserves the motility, integrity, and oxidative status of the sperm. On the other hand, a 4% concentration of G would be at the lower limit of toxicity. Interestingly, the 2% and 3% concentrations of G gave numerically poorer results compared to the control diluent for all the parameters studied, but these differences were not significant.

The 2.5% concentration of G seems to be the optimal concentration of G to preserve the functionality, integrity

Table 9. Spermatozoal parameters after the second thawing in the three sperm cell concentrations with (CLC+TLC) or without (NT) treatment (T1-Exp 2) (P<0.05; n=18 ejaculates)

Parameter [‡]	1x10 ⁶ Spermatozoa/mL		5x10 ⁶ Spermatozoa/mL		10x10 ⁶ Spermatozoa/mL	
	NT	CLC+TLC	NT	CLC+TLC	NT	CLC+TLC
VIAB/ACRO (%)	14.05±1.07 ^a	29.08±1.09 ^b	20.56±0.98 ^a	32.26±1.74 ^b	20.97±1.00 ^a	27.37±1.35 ^b
ROS (%)	43.19±1.62 ^a	32.93±1.04 ^b	28.92±1.83 ^a	21.48±1.59 ^b	27.35±1.61 ^a	22.85±1.59 ^b
MOT (%)	6.07±0.37 ^a	13.00±0.88 ^b	8.99±0.21 ^a	18.50±0.41 ^b	8.81±0.21 ^a	11.45±0.26 ^a
PROG (%)	5.03±0.36 ^a	11.79±1.07 ^b	7.48±0.31 ^a	11.94±0.59 ^b	7.34±0.31 ^a	8.83±0.39 ^a

[‡]VIAB/ACRO denotes the percentage of viable spermatozoa possessing an intact acrosome; ROS (%) denotes the percentage of living spermatozoa that had undergone oxidation due to reactive oxygen species; MOT (%) denotes the percentage of spermatozoa exhibiting motility; PROG (%) denotes the percentage of spermatozoa exhibiting progressive motility

and oxidative balance of equine spermatozoa through two cycles of cryopreservation. Although the results of the present study are similar to those of another study^[36] where the best concentration of G ranged from 2.4 to 2.8%, other researchers^[17,37] found the most suitable concentration of G was 4.0%. In the same context, Hoffmann et al.^[38] reported that concentrations of 2-3% of the cryoprotectants with freezing extender showed the highest motility rates after freeze-thaw, both for 'good' and 'poor' freezers. On the other hand, Gonzalez-Castro et al.^[39] found that G concentration (2, 4, 6 or 8%) did not affect post-thaw motility. The discrepancies in results of various studies make it difficult to establish an optimal concentration of G. Different results across studies may be due to the variation in diluent composition, quality control in the laboratory, individual stallion effects, or other factors.

The effect of final sperm cell concentration on motility and integrity of twice-frozen equine spermatozoa was investigated in the second study. Three final concentrations of spermatozoa (10×10^6 , 5×10^6 , 1×10^6 spermatozoa/mL) were compared, and no differences in sperm motility, integrity, or oxidative damage were observed between sperm concentrations of 10×10^6 or 5×10^6 spermatozoa/mL. It is common for most straws of stallion semen to possess between 100 and 300×10^6 spermatozoa/mL, so the dilution rates used in the present study allowed the production of 10-20, 20-40, or 30-60 ICSI-dosed straws from a single conventional AI straw having 100×10^6 , 200×10^6 , or 300×10^6 spermatozoa/mL, respectively.

The lowest sperm concentration evaluated (1×10^6 spermatozoa/mL) would enable production of between 100 and 300 ICSI straws from a single conventional AI straw. However, the control diluent without the added CLC+TLC treatment yielded poorer sperm motility, integrity and oxidative status in comparison with the other two concentrations. No definitive explanation for this result is known. Regardless of the reasons that may explain our data, it would be interesting to develop a semen treatment capable of improving the results by increasing the resistance of spermatozoa to refreezing.

Given the paucity of cholesterol in the plasma membrane of the equine spermatozoon and the increase of oxidative stress caused by accumulated ROS, we hypothesized that a pre-refreezing treatment of semen based on cholesterol and an antioxidant such as α -tocopherol may be beneficial in the prevention of cryoinjury generated by reactive oxygen species (ROS). Treatment of sperm with a combination of cholesterol and α -tocopherol loaded into cyclodextrin (CLC+TLC) at the half dose of 2.5 mg CLC per 100×10^6 spermatozoa and 0.05 mM TLC per 100×10^6 spermatozoa before refreezing allowed, under the conditions of the current experiment, a considerable

improvement in sperm motility, integrity and oxidative damage in the three dilutions studied (with the exception of the motility in the 1:100 dilution [1×10^6 spermatozoa/mL]).

The lowest sperm cell concentration studied, which is of the greatest interest in terms of production of ICSI straws, resulted in a marked improvement in the percentages of motile (MOT), progressive (PROG) and viable spermatozoa with intact acrosomes. These results could be explained by the protective and the stabilizing action of cholesterol on the equine sperm plasma membrane that is poor in cholesterol^[8], as well as by the antioxidant action of α -tocopherol - particularly for cells suffering with disruption of their metabolism and free radical elimination mechanism. Our result agrees with that of other studies^[40,41]. Specifically, α -tocopherol can stop the lipid peroxidation chain reaction in biomembranes, protecting the cell from damage to the plasma and acrosomal membranes^[42]. In addition, CLCs are believed to prevent the rearrangement of phospholipids by cholesterol addition and to increase membrane fluidity at low temperatures^[43].

In addition to the protective action of cholesterol and α -tocopherol, the effectiveness of this treatment can also be explained by the ability of methyl- β -cyclodextrin to increase the solubility (in an aqueous medium) of cholesterol and α -tocopherol (lipid molecules) thereby improving their penetration into the sperm plasma membrane. The beneficial effects of CLC and TLC observed in the current study agree with other previous investigations^[42-44]. Some reports revealed that CLC, either loaded with cholesterol or unloaded but in the presence of a high cholesterol content diluent, improved post-thaw motility, viability, and fertility but inhibited post-thaw acrosome reaction of equine and bovine semen^[45-47].

To further improve results, it would be interesting to enhance this treatment in the future by optimizing the dose and duration of treatment immediately before both the first and second freezing. It would also be interesting to evaluate the benefit of this treatment without re-centrifugation before refreezing. More sperm parameters to include in the future, DNA integrity, is very crucial in term of ICSI and in IVF procedures.

Refreezing of equine spermatozoa in the control diluent is possible, but with a considerable reduction in sperm quality parameters (motility, integrity and oxidative status). Treatment of spermatozoa before refreezing with cholesterol and α -tocopherol loaded cyclodextrins seems to improve all quality parameters of refrozen sperm cells. Further investigations into the exact biological mechanism and the separate effect of each substance (cholesterol and α -tocopherol) on equine sperm cryopreservation is warranted.

DECLARATIONS

Data Availability Statement: The data that support the findings of this study are available from the corresponding author (NM) upon reasonable request.

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RESEARCH ARTICLE

Molecular Characterization, Virulence Determinants, Antimicrobial Resistance, and Genotyping of *Streptococcus uberis* Isolated from Mastitic Dairy Cows in the Aegean Region of Türkiye

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Abstract

Bovine mastitis remains one of the most prevalent and economically damaging diseases of dairy herds, and *Streptococcus uberis* is a leading environmental pathogen implicated in both clinical and subclinical infections. In this study, we characterized *S. uberis* isolates from mastitis-suspected dairy cows in the Aegean Region of Türkiye using phenotypic and molecular identification, virulence and antimicrobial resistance (AMR) gene profiling, antimicrobial susceptibility testing, and ERIC-PCR genotyping. Overall, 167 milk samples were cultured and *S. uberis* was isolated from 11 samples (6.6%). Identification was performed with VITEK® 2 and confirmed by 16S rRNA PCR (1.400 bp). Virulence genes were widely distributed, including capsule genes (*hasB* 91%, *hasC* 82%, *hasA* 73%) and adhesion/invasion-related loci (*gapC* and *oppF* 82% each, *skc* 91%, *sua* 64%, *cfu* 73%), whereas *lbp* showed lower prevalence (27%). AMR gene screening revealed high carriage of penicillin-binding protein genes (*pbp1A*, *pbp2A*, *pbp2B* 100%; *pbp1B* 90.9%), *blaTEM* (72.7%), *blaZ* (9.1%), *ermA/ermB* (100%), *tetM* (54.5%), and *gyrA/parC* (27.3% each). Phenotypically, resistance was frequent, notably to tetracycline (100%) and penicillin G (63.6%), with heterogeneous patterns to other agents, indicating multidrug resistance. ERIC-PCR demonstrated marked genetic heterogeneity, identifying two small clusters (2 and 3 isolates) and six unique profiles at a 70% similarity cut-off, consistent with multiple environmental sources rather than a single dominant clone. These findings underscore the need for improved environmental hygiene and susceptibility-guided therapy, and support further molecular surveillance of circulating strains and resistance mechanisms.

Keywords: Antimicrobial resistance, Bovine mastitis, ERIC-PCR, *Streptococcus uberis*, Virulence genes

INTRODUCTION

Bovine mastitis remains one of the most common and economically detrimental diseases affecting dairy herds worldwide. It reduces milk yield, alters milk composition, increases culling rates, and results in substantial therapeutic and veterinary expenses. Annual global losses attributable to mastitis are estimated to reach billions of dollars, driven largely by reduced productivity, discarded milk, treatment costs, and premature culling [1-4]. Subclinical mastitis is particularly challenging because it often remains unnoticed, leading to chronic inflammation and sustained milk losses over time.

Mastitis-associated pathogens are broadly classified as contagious or environmental. Contagious agents primarily spread during milking; however, improved milking hygiene practices and dry-cow therapy have decreased

their relative importance in many herds [5,6]. In parallel, environmental pathogens have become increasingly predominant, emphasizing the role of housing conditions, bedding, manure contamination, and overall farm management in mastitis epidemiology.

Among environmental pathogens, *Streptococcus uberis* is recognized as a major etiological agent of bovine mastitis [1]. Its prevalence varies substantially across regions and production systems, ranging from approximately 15-40% in Europe and North America and reaching higher levels in pasture-based systems [5,7,8]. In Türkiye, *S. uberis* has been increasingly reported in dairy herds, and humid, pasture-influenced areas such as the Aegean Region may provide favorable conditions for persistence and transmission [9]. The pathogenic success of *S. uberis* is linked to a diverse set of virulence factors, including capsule-associated determinants that support immune evasion, adhesion-



related factors that facilitate colonization and persistence within the mammary gland, and additional components involved in tissue invasion and cytolytic activity^[9,10].

Antimicrobial therapy remains a cornerstone of mastitis control; nevertheless, treatment failure is increasingly reported due to escalating antimicrobial resistance. In *S. uberis*, β -lactam resistance has been associated with alterations in penicillin-binding proteins (e.g., *pbp1A*, *pbp2A*, *pbp2B*) and, less frequently, β -lactamase-related mechanisms (e.g., *blaTEM*, *blaZ*)^[11]. Macrolide resistance is largely mediated by *ermA* and *ermB*, whereas tetracycline resistance is frequently linked to *tetM*. Emerging fluoroquinolone resistance has also been associated with mutations in key target genes such as *gyrA* and *parC*^[12-15]. Genotyping studies frequently demonstrate high genetic diversity among *S. uberis* isolates, supporting the concept that many infections arise from environmental reservoirs rather than direct cow-to-cow^[6]. Therefore, the present study aimed to characterize *S. uberis* isolates obtained from mastitis-suspected (CMT-positive) dairy cows in the Aegean Region of Türkiye through phenotypic and molecular identification, virulence gene profiling, antimicrobial susceptibility testing, detection of resistance determinants, and assessment of genetic diversity using ERIC-PCR.

MATERIAL AND METHODS

Ethical Statement

All experimental procedures were approved by the Local Ethics Committee of the Bornova Veterinary Control Institute (Date: 01.04.2025, Decision No: 2025/2).

Sample Size Justification and Bacterial Identification

Assuming an expected prevalence of approximately 10% for *S. uberis* in mastitic milk, the sample size was calculated under a binomial framework to estimate prevalence with acceptable precision^[16]. A total of 167 CMT-positive quarter milk samples were collected from mastitis-

suspected dairy cows located in eight provinces of the Aegean Region of Türkiye, namely İzmir, Aydın, Manisa, Muğla, Denizli, Uşak, Kütahya, and Afyonkarahisar.

A descriptive, cross-sectional sampling strategy was used. Seven dairy farms were included from each province, resulting in a total of 56 farms. Three CMT-positive quarter milk samples were collected from each farm, except for one farm in Afyonkarahisar, from which two samples were obtained. Thus, the final sample size consisted of 167 milk samples. Quarter selection was based on the California Mastitis Test (CMT), and samples were collected from quarters with a CMT score $\geq 1+$ ^[17,18].

Samples were submitted to the Bornova Veterinary Control Institute. Each sample was cultured on 5% ovine blood agar (Oxoid®, UK) and incubated aerobically at 37°C for 24-48 h^[19,20]. After incubation, colonies with streptococcal morphology were selected and examined by Gram staining; isolates showing Gram-positive cocci in chains and a negative catalase reaction with 3% H₂O₂ were considered consistent with streptococci^[5,20]. Isolates were identified using the VITEK® 2 Compact system (bioMérieux, France) with GP identification cards according to the manufacturer's instructions^[9,19]. Genomic DNA was extracted from purified *S. uberis* isolates using the High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. The extracted DNA samples were stored at -20°C until further PCR analysis. Molecular confirmation was performed by PCR targeting the 16S rRNA gene using published primers (Forward, 5'-GAGAGTTTGATCCTGGCTCAGGA-3'; Reverse, 5'-CGGGTGTACAACTCTCGTGGT-3'); the expected 1,400bp amplicon was interpreted as positive^[20].

Detection of Virulence Genes

Virulence-associated genes were screened in all *S. uberis* isolates by PCR using genomic DNA extracted as described above. The following loci were targeted: *hasA*, *hasB*, *hasC*, *gapC*, *oppF*, *skc*, *sua*, *cfu* and *lbp*, which are associated with adhesion, immune evasion, and persistence in the bovine

Table 1. Primers used for amplification of virulence-associated genes in *S. uberis* isolates

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon (bp)	Annealing Temperature (°C)	Ref.
<i>cfu</i>	TATCCCGATTTCAGCCTAC	CCTGGTCAACTGTGCAACTG	205	59	[20]
<i>hasA</i>	GAAAGGTCTGATGCTGAT	TCATCCCCTATGCTTACAG	600	58	[20]
<i>hasB</i>	TCTAGACGCCGATCAAGC	TGAATTCCYATGCGTCGATC	300	58	[20]
<i>hasC</i>	TGCTTGTTGACGATTGTGATG	GTCCAATGATAGCAAGGTACAC	300	58	[20]
<i>gapC</i>	GCTCCTGGTGGAGATGATGT	GTCACCAGTGTAAGCGTGGA	200	55	[20]
<i>lbp</i>	CGACCTTCAGATTGGATTC	TAGCAGCATCACGTTCTTCG	698	53	[20]
<i>oppF</i>	GGCCTAACCAAAACGAAACA	GGCTCTGGAATTGCTGAAAG	419	54	[20]
<i>skc</i>	CTCCTCTCCAACAAAGAGG	GAAGGCCTTCCCCTTTGAAA	800	52	[20]
<i>sua</i>	ACGCAAGGTGCTCAAGAGTT	TGAACAAGCGATTCGTCAAG	776	58	[20]

mammary gland ^[9,20]. Published primer sets and expected amplicon sizes were used ^[20] (Table 1).

PCR was performed in 25 µL reaction volumes containing Xpert Fast Hotstart Mastermix (2×, GRiSP®, Portugal), gene-specific primers, nuclease-free water, and template DNA. All PCR assays were performed as separate single-target reactions for each gene; no multiplex PCR protocol was used in this study. Cycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 35-40 cycles of 95°C for 30 s, gene-specific annealing for 30 s, and extension at 72°C for 30-60 s, with a final extension at 72°C for 5 min. Amplicons were resolved by electrophoresis on 1.5% agarose gels and visualized under UV illumination. The presence of each gene was recorded based on the expected product size (Table 1).

Detection of Antimicrobial Resistance Genes

Antimicrobial resistance (AMR) genes were screened in all *S. uberis* isolates by PCR using genomic DNA prepared as described above. Eleven resistance-associated loci representing major antimicrobial classes were targeted: penicillin-binding protein genes (*pbp1A*, *pbp1B*, *pbp2A*, *pbp2B*), β-lactamase genes (*blaTEM*, *blaZ*), macrolide resistance genes (*ermA*, *ermB*), the tetracycline resistance gene (*tetM*), and quinolone resistance-associated genes (*gyrA*, *parC*) ^[13,21-23].

However, for *pbp*, *gyrA*, and *parC*, PCR positivity was interpreted only as the presence of the corresponding target locus/amplicon. Since sequence analysis was not performed, resistance-conferring mutations or allelic variations in these loci could not be demonstrated.

PCR was performed in 25 µL reaction volumes containing Xpert Fast Hotstart Mastermix (2×, GRiSP®, Portugal), gene-specific primer pairs, nuclease-free water, and template DNA. All PCR assays were performed as separate

single-target reactions for each gene; no multiplex PCR protocol was used in this study. Cycling conditions followed published protocols: initial denaturation at 95°C for 3 min; 35-40 cycles of 95°C for 30 s, gene-specific annealing for 30 s, and 72°C extension for 30-60 s; and a final extension at 72°C for 5 min ^[18,21,24-26]. Amplicons were resolved on 1.5% agarose gels and visualized under UV illumination. The presence of each gene was recorded according to the expected product size. Primer sequences and amplicon sizes are presented in Table 2.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of all *S. uberis* isolates was determined by the disk diffusion method on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines ^[29]. Bacterial suspensions were prepared in sterile saline and adjusted to 0.5 McFarland, and plates were inoculated by swabbing the entire agar surface. Commercial antimicrobial disks (Oxoid®, UK) were applied, and plates were incubated aerobically at 37°C for 18-24 h.

The antimicrobial disks tested were penicillin G (10 units), ampicillin (10 µg), erythromycin (15 µg), tetracycline (30 µg), oxytetracycline (30 µg), cefquinome (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), and chloramphenicol (30 µg). After incubation, inhibition zone diameters were measured in millimetres. Isolates were categorized as susceptible, intermediate, or resistant according to CLSI M100, 34th edition, 2024, where applicable. For antimicrobial agents without directly applicable CLSI interpretive criteria for *S. uberis*, interpretation was performed using published criteria from previous studies on mastitis-associated streptococci, as clearly indicated in Table 4 ^[26]. *Streptococcus pneumoniae* ATCC 49619 was used for quality control according to CLSI

Table 2. Primers used for amplification of antimicrobial resistance genes in *S. uberis* isolates

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon (bp)	Annealing Temperature (°C)	Ref.
<i>pbp1A</i>	GGCGACATCTGGATGAAAT	GCCATTGTTCCAACATAATCA	718	52	[21]
<i>pbp1B</i>	CTTTGGCGGTTTGCTAGATG	GGATGGCGTTGGCTAGATTA	890	52	[21]
<i>pbp2A</i>	AGGGCTTGTTGGTCGTGTTA	CGGTCTTGTTAAACCGATCC	734	52	[21]
<i>pbp2B</i>	CTATGTCGGGCTTGTCCTCGT	TGGCAACAGCTACTTCAGGA	879	52	[21]
<i>blaZ</i>	TAAGAGATTGTCCTATGCTT	TTAAAGTCTTACCGAAAGCAG	377	52	[23]
<i>blaTEM</i>	ATGAGTATTCAACATTTTCGTG	TTACCAATGCCTAATCAGTGAG	860	58	[27]
<i>ermA</i>	TCAGGAAAAGGACATTTTACC	ATACTTTTTGTAGTCCTTCTT	432	58	[28]
<i>ermB</i>	TTTTTGAAAGCCATGCGTCT	CTGTGGTATGGCGGTAAGT	201	60	[13]
<i>tetM</i>	GTGGACAAAGGTACAACGAG	CGGTAAAGTTTCGTCACACAC	406	55	[25]
<i>gyrA</i>	CGATGTCGGTCATTGTTG	ACTTCCGTCAGGTTGTGC	496	50	[22]
<i>parC</i>	TGGGTTGAAGCCGGTTCA	CAAGACCGTTGGTTCTTTC	361	58	[24]

Table 3. Provincial and farm-level distribution of milk samples and *S. uberis* isolates

Province	No. of Farms Sampled	No. of Milk Samples	No. of <i>S. uberis</i> -Positive Farms	No. of <i>S. uberis</i> -Positive Isolates	Sample-Level Positivity (%)	Farm-Level Positivity (%)
İzmir	7	21	3	3	14.3	42.9
Aydın	7	21	2	2	9.5	28.6
Manisa	7	21	2	2	9.5	28.6
Muğla	7	21	1	1	4.8	14.3
Denizli	7	21	1	1	4.8	14.3
Uşak	7	21	1	1	4.8	14.3
Kütahya	7	21	0	0	0.0	0.0
Afyonkarahisar	7	20	1	1	5.0	14.3
Total	56	167	11	11	6.6	19.6

recommendations [29]. Phenotypic resistance results were compared descriptively with AMR-associated locus profiles.

Genotyping by ERIC-PCR

Genetic relatedness among the isolates was assessed by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using the ERIC-2 primer (5'-AAGTAAAGTGA CTGGGGTGAGCG-3'), and amplification was carried out following a previously outlined procedure [30]. Briefly, a 25 µL ERIC-PCR reaction mixture was prepared containing 1× PCR buffer, 2.5 mM MgCl₂, 200 µM of each dNTP, 2.5U Taq DNA polymerase, 25 pmol primer, and 5 µL template DNA. Thermal cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 3 min, with a final extension at 72°C for 7 min [30]. Amplification products were separated on 1.5% agarose gels containing ethidium bromide (2 µg/mL) and visualized under UV illumination. ERIC-PCR banding patterns were analyzed to construct dendrograms using the unweighted pair group method with arithmetic averages (UPGMA), and genetic relatedness was interpreted using a 70% similarity cut-off.

RESULTS

Isolation and identification of *Streptococcus uberis*

A total of 167 CMT-positive quarter milk samples collected from mastitis-suspected dairy cows in eight provinces of the Aegean Region of Türkiye were examined. *S. uberis* was isolated from 11 samples, corresponding to an overall sample-level positivity rate of 6.6% and a farm-level positivity rate of 19.6% (11/56).

Among the 11 *S. uberis*-positive samples, three isolates were obtained from three different farms in İzmir, two isolates from two farms in Aydın, and two isolates from two farms in Manisa. In addition, one isolate each was recovered from a single farm in Muğla, Denizli, Uşak, and Afyonkarahisar. No *S. uberis* isolate was recovered from Kütahya. *S. uberis* was isolated from 11 samples (6.6%) (Table 3).

On 5% ovine blood agar, isolates produced 1-3 mm, opaque S-type colonies. Gram staining revealed Gram-positive cocci in chains, and all isolates were catalase-negative. Species identification was performed using the

Table 4. Antimicrobial susceptibility profiles of *S. uberis* isolates (n = 11)

Antimicrobial Agent	Disk Content	Interpretive Reference	Resistant (n)	Susceptible (n)	Resistance (%)	Detected Related AMR Genes/ loci
Penicillin G	10 units	[29]	7	4	63.6	<i>pbp1A</i> , <i>pbp1B</i> , <i>pbp2A</i> , <i>pbp2B</i> , <i>blaTEM</i> , <i>blaZ</i>
Ampicillin	10 µg	[29]	1	10	9.1	<i>pbp1A</i> , <i>pbp1B</i> , <i>pbp2A</i> , <i>pbp2B</i> , <i>blaTEM</i> , <i>blaZ</i>
Erythromycin	15 µg	[26,29]	5	6	45.5	<i>ermA</i> , <i>ermB</i>
Tetracycline	30 µg	[26,29]	11	0	100.0	<i>tetM</i>
Oxytetracycline	30 µg	[26]	10	1	90.9	<i>tetM</i>
Cefquinome	30 µg	[26]	11	0	100.0	<i>pbp1A</i> , <i>pbp1B</i> , <i>pbp2A</i> , <i>pbp2B</i> , <i>blaTEM</i> , <i>blaZ</i>
TMP-SMX	1.25/23.75 µg	[26,29]	11	0	100.0	Not investigated
Chloramphenicol	30 µg	[26,29]	11	0	100.0	Not investigated

VITEK® 2 GP system and molecular confirmation was conducted by PCR targeting the 16S rRNA gene, yielding the expected 1.400 bp amplicon in all 11 isolates (Fig. 1).

The overall sample-level positivity rate was 6.6% (11/167; 95% Wilson CI: 3.7-11.4%). Provincial positivity rates ranged from 0.0% to 14.3%, with the highest rate observed in İzmir (3/21; 14.3%; 95% CI: 5.0-34.6%), followed by Aydın and Manisa (2/21 each; 9.5%; 95% CI: 2.7-28.9%). Lower rates were detected in Muğla, Denizli, Uşak, and Afyonkarahisar, whereas no *S. uberis* isolate was recovered from Kütahya. Although numerical differences were observed among provinces, the difference in sample-level positivity was not statistically significant by chi-square test ($\chi^2 = 4.52$, $df = 7$, $P = 0.719$). Because of the small number of positive isolates and low expected cell counts, the result was also checked using the Fisher-Freeman-Halton exact test with Monte Carlo simulation, which similarly showed no significant provincial difference ($P = 0.778$).

At the farm level, *S. uberis* was detected in 11 of 56 sampled dairy farms, corresponding to a farm-level positivity rate of 19.6% (95% Wilson CI: 11.3-31.8%). Farm-level positivity varied from 0.0% in Kütahya to 42.9% in İzmir. However, this variation was not statistically significant among provinces either by chi-square test ($\chi^2 = 5.32$, $df = 7$, $P = 0.621$) or by Fisher-Freeman-Halton exact test with Monte Carlo simulation ($P = 0.783$). Therefore, although İzmir showed the highest numerical positivity, the present data do not support a statistically significant provincial clustering of *S. uberis* positivity.

Distribution of Virulence Genes

All *S. uberis* isolates were screened for virulence-associated genes. Capsular synthesis-related genes were frequently detected (*hasB* 91%, *hasC* 82%, *hasA* 73%). Adhesion-associated genes were also common (*gapC* 82%, *oppF* 82%, *sua* 64%). The *skc* gene was present in 91% of isolates,

whereas *cfu* was detected in 73%. The *lbp* gene showed the lowest prevalence (27%). Overall, most isolates carried multiple virulence determinants (Fig. 2).

Detection of Antimicrobial Resistance Genes

All *S. uberis* isolates were screened for 11 AMR-associated genes. Penicillin-binding protein genes were frequently detected: *pbp1A*, *pbp2A*, and *pbp2B* were present in all isolates (100%, 11/11), whereas *pbp1B* was detected in 90.9% (10/11). Among β -lactamase genes, *blaTEM* was identified in 72.7% (8/11), while *blaZ* was detected in 9.1% (1/11). Macrolide resistance genes (*ermA* and *ermB*) were detected in all isolates (100%, 11/11), and the tetracycline resistance gene *tetM* was present in 54.5% (6/11). Quinolone resistance-associated genes *gyrA* and *parC* were each detected in 27.3% (3/11). Overall, most isolates carried multiple AMR determinants (Fig. 3).

Antimicrobial Susceptibility Results

Antimicrobial susceptibility testing demonstrated heterogeneous resistance patterns among the *S. uberis* isolates (Table 4). Resistance to penicillin G was observed

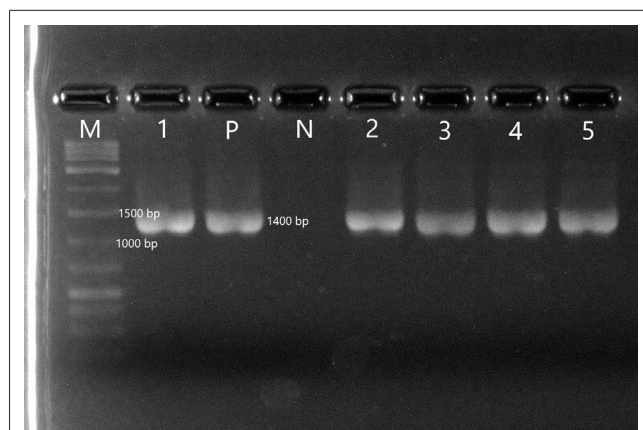


Fig 1. Agarose gel electrophoresis image of PCR amplification of the 16S rRNA gene, showing the expected 1,400bp amplicon. Lanes 1-5: test samples/isolates, P: positive control, N: negative control, M: DNA marker

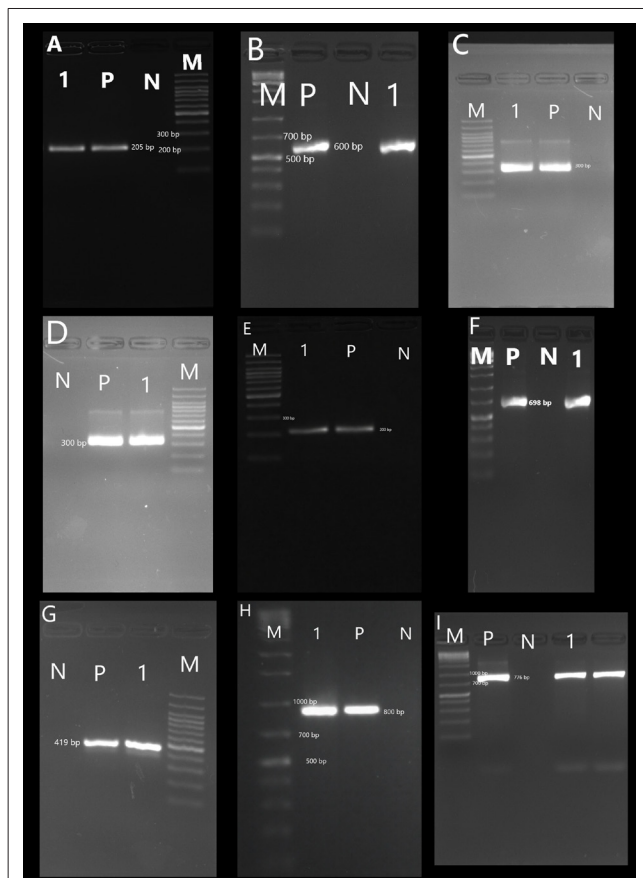


Fig 2. Agarose gel electrophoresis images of PCR amplicons for the virulence genes investigated in this study. A: *cfu* (205bp), B: *hasA* (600bp), C: *hasB* (300bp), D: *hasC* (300bp), E: *gapC* (200bp), F: *lbp* (698bp), G: *oppF* (419bp), H: *skc* (800bp), I: *sua* (776bp). P: positive control, N: negative control, 1: test sample/isolate, M: DNA marker

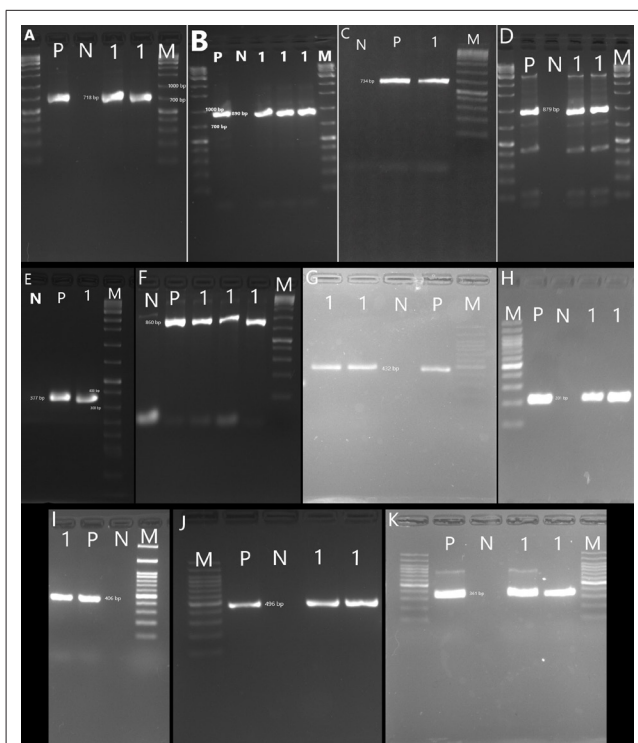


Fig 3. Agarose gel electrophoresis images of PCR amplicons targeting β -lactam resistance, macrolide resistance, tetracycline resistance, and quinolone resistance-associated genes. Panels: A: *pbp1A*, 718bp; B: *pbp1B*, 890bp; C: *pbp2A*, 734bp; D: *pbp2B*, 879bp; E: *blaZ*, 377bp; F: *blaTEM*, 860bp; G: *ermA*, 432bp; H: *ermB*, 201bp; I: *tetM*, 406bp; J: *gyrA*, 496bp; K: *parC*, 361bp. P: positive control; N: negative control; 1: test isolate/sample; M: DNA size marker

in 63.6% (7/11) of isolates, whereas most isolates remained susceptible to ampicillin (90.9%, 10/11). All isolates were resistant to tetracycline, cefquinome, trimethoprim-sulfamethoxazole, and chloramphenicol (each 100%, 11/11), and resistance to oxytetracycline was also high (90.9%, 10/11). Erythromycin resistance was detected in 45.5% (5/11). Collectively, the susceptibility profiles indicate a high prevalence of multidrug resistance in the study population (Table 4). Molecular results indicate PCR detection of related AMR genes/loci in the isolate collection and do not necessarily indicate phenotypic resistance in each isolate.

ERIC-PCR Genotyping Results

ERIC-PCR fingerprinting generated clear and reproducible banding patterns (Fig. 4). At a 70% similarity cut-off, two clusters were identified: one comprising two isolates (RG1 and RG2) and another comprising three isolates (RH1-RH3). The remaining six isolates (RA-RF) exhibited unique profiles and did not cluster with other isolates at this threshold. Overall, ERIC-PCR indicated marked genetic heterogeneity among the isolates, consistent with the presence of multiple genotypes rather than a single dominant clone (Fig. 4).

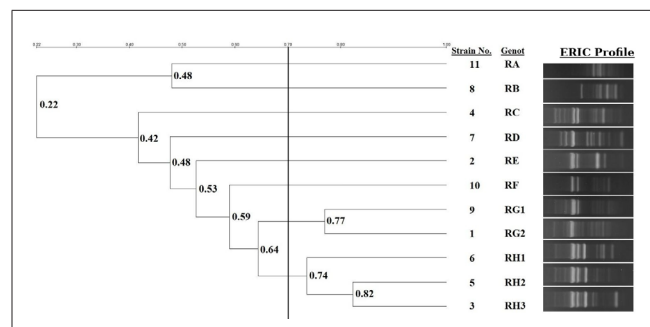


Fig 4. ERIC-PCR banding patterns and corresponding dendrogram of *S. uberis* isolates

DISCUSSION

This study determined a *S. uberis* isolation rate of 6.6% from 167 milk samples collected from mastitis-suspected (CMT-positive) dairy cows in the Aegean Region of Türkiye. This rate is close to the lower bound of the 10-25% range reported in some previous studies from Türkiye and may reflect differences in herd type, sampling strategy, seasonal/environmental conditions, and management practices [9]. A previous study from Aydın Province, located within the Aegean Region, reported the detection of virulence genes in *S. uberis* isolates from bovine mastitis cases [9]. The presence of this earlier regional report supports the relevance of *S. uberis* as a mastitis-associated pathogen in the Aegean Region. However, the present study differs by including isolates obtained from multiple provinces of the region and by evaluating virulence-associated genes together with antimicrobial susceptibility, resistance-associated loci, and ERIC-PCR genotyping. Therefore, our findings expand the available regional data beyond a single-province observation. Globally, *S. uberis* prevalence has been reported to vary widely, ranging approximately from 15% to 40% in Europe and North America, and reaching higher levels in pasture-based systems [5,6,8]. Therefore, the relatively low isolation rate observed in the present study does not diminish the regional relevance of this pathogen; rather, its virulence potential and treatment challenges highlight that it remains economically and clinically important in mastitis management.

The high detection frequencies of virulence-associated genes in our isolates suggest that *S. uberis* circulating in the region may possess substantial genetic capacity for mammary gland colonization and persistence. In particular, the capsule synthesis-related genes *hasA* (73%), *hasB* (91%), and *hasC* (82%) were widely distributed. This distribution is in line with previous studies from Türkiye and other regions, which have shown a high prevalence of capsule-associated genes and support the importance of the capsule as a conserved virulence determinant involved in immune evasion and persistence [9,20,30-32].

The detection of adhesion-associated genes *oppF* (82%), *gapC* (82%), and *sua* (64%) indicates a strong potential for early adherence to the mammary epithelium and persistence during intramammary infection. These findings are consistent with previous reports demonstrating the presence of adhesion-related genes in *S. uberis* isolates and support their possible role in colonization and persistence during infection [9]. In addition, the detection of *sua* and *gapC* may indicate a potential association with a prolonged clinical course and a tendency toward chronic mastitis [33]. Taken together, the adhesion gene patterns observed here suggest that, despite its predominantly environmental origin, *S. uberis* may establish persistent infections in mammary tissue.

Virulence determinants associated with tissue invasion and cellular damage were also frequently detected, including *skc* (91%) and *cfu* (73%). This profile suggests a notable virulence potential among the isolates. The high prevalence of *cfu* in the present study is in agreement with previous findings reporting this gene at high frequency among *S. uberis* isolates, further supporting its potential role in the pathogenesis of bovine mastitis [9,20]. In contrast, the relatively low prevalence of *lbp* (27%) may reflect strain-to-strain variability in iron acquisition mechanisms and possible regional differences in adaptation. Overall, the co-occurrence of multiple virulence genes in most isolates indicates a multifactorial pathogenic profile and further supports the continuing importance of *S. uberis* as a mastitis pathogen [9,10,19].

Phenotypic antimicrobial susceptibility results indicated a high resistance burden, particularly against tetracyclines and several other agents. These findings highlight the limitations of empirical therapy in mastitis cases and underscore the need for treatment strategies guided by isolation, identification, and susceptibility testing. For β -lactams, resistance to penicillin (63.6%) together with largely preserved susceptibility to ampicillin suggests that resistance may not be explained solely by classical β -lactamase presence, but could involve target alterations and/or differences in breakpoint interpretation. In this context, the presence of PBP-related genes alone may be insufficient to predict phenotype; rather, allelic variation/mutations and expression or regulatory differences are likely to be more decisive [21,32-35]. Local data from Türkiye reporting frequent detection of *pbp* genes, yet incomplete concordance with phenotypic resistance, are compatible with this interpretation [36,37].

For macrolides, erythromycin resistance was 45.5%, whereas all isolates carried *ermA/ermB*, indicating that gene presence and phenotypic resistance may not fully coincide. This discrepancy may be related to inducible expression of *erm* genes, regulatory variation, or contributions of non-*erm* mechanisms such as efflux

systems [13,14,22,32,38,39]. Similarly, the observation of 100% phenotypic tetracycline resistance despite *tetM* positivity of 54.5% suggests that additional genes (e.g., *tetO/tetL*) or efflux mechanisms may contribute. Detection of fluoroquinolone target gene alterations (*gyrA* and *parC*) at 27.3% may reflect a limited but present selection pressure in the region; given their potential contribution to treatment failure, these markers warrant continued monitoring [14,22]. Therefore, the molecular findings for *pbp*, *gyrA*, and *parC* should be interpreted with caution, as sequencing was not performed to confirm resistance-associated mutations.

Finally, ERIC-PCR genotyping revealed marked genetic heterogeneity among the isolates. As shown in Fig. 4, the presence of two small clusters at the 70% similarity threshold (RG1-RG2 and RH1-RH3) together with multiple unique profiles indicates that cases were associated with circulation of multiple genotypes rather than a single dominant clone. This finding is consistent with the prevailing concept that *S. uberis* mastitis frequently arises from environmental sources via multiple introductions and that substantial molecular diversity may occur within and between herds [22,40].

This study has some limitations. First, although the study provides regional data on *S. uberis* isolates from mastitic dairy cows in the Aegean Region of Türkiye, it was designed as a descriptive, cross-sectional study and should not be interpreted as a randomized prevalence survey representing all dairy herds in the region. Second, the methods used, including bacterial isolation, PCR-based gene screening, antimicrobial susceptibility testing, and ERIC-PCR, are conventional and do not provide the resolution of sequencing-based approaches. In particular, sequence analysis of *pbp*, *gyrA*, and *parC* loci was not performed; therefore, resistance-associated mutations or allelic variations could not be confirmed. Further studies using larger isolate collections and whole-genome sequencing are needed to clarify the epidemiology, virulence potential, and resistance mechanisms of *S. uberis* in Türkiye.

In conclusion, this study demonstrated that *S. uberis* isolates recovered from bovine mastitic dairy cows in the Aegean Region of Türkiye harbor a substantial repertoire of virulence-associated genes, exhibit pronounced antimicrobial resistance patterns, and display marked genetic heterogeneity. Collectively, these findings support the role of *S. uberis* as an important pathogen in environmentally acquired intramammary infections and suggest a considerable potential for persistence within the mammary gland. The high resistance observed to several commonly used antimicrobials underscores the need to curb indiscriminate antibiotic use and highlights the importance of susceptibility-guided therapy. Although

the study does not provide methodological novelty, it contributes regional baseline data by evaluating phenotypic characteristics, virulence-associated genes, antimicrobial susceptibility profiles, resistance-associated loci, and ERIC-PCR genotypes of *S. uberis* isolates from mastitic dairy cows in the Aegean Region of Türkiye. Accordingly, improving environmental hygiene, implementing appropriate herd management practices, and selecting antimicrobials based on susceptibility testing should be prioritized for effective mastitis control. Further molecular and genomic investigations are warranted to better elucidate the epidemiology of circulating strains and the underlying resistance mechanisms.

DECLARATIONS

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RESEARCH ARTICLE

SIR-Based Modeling of Viral Infectious Diseases in Farm Animals

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Abstract

Throughout history, epidemic diseases have had profound impacts on societies, causing serious problems in public health as well as adverse effects on food security and animal health. When real-time data are processed through mathematical and statistical models, it becomes possible to evaluate the course and effects of an outbreak. In this study, the progression of a disease was simulated using the SIR (Susceptible-Infectious-Recovered/Removed) mathematical model, which is one of the most widely used models for epidemic analysis, by incorporating transmission and recovery rates. For this purpose, three different simulations were performed on a herd consisting of N=1000 animals over a 10-day period, and the expected numbers of susceptible, infected, and recovered animals were obtained. Evaluation of the different simulation rates revealed that when the contact rate and recovery rate were reduced, the number of susceptible animals remained constant, while the number of infected animals increased and the number of recovered animals decreased after 10 days. This model provides a valuable preliminary assessment and decision-support tool for epidemic management and control strategies. Furthermore, the development of such models is crucial for early detection and effective intervention in future outbreaks.

Keywords: Epidemic Diseases, Herd, SIR model, Viral diseases

INTRODUCTION

Epidemics have historically affected societies significantly, causing major health issues. Various strategies have been used to comprehend, manage, and avert outbreaks, with mathematical and statistical modeling offering crucial insights into the dynamics, effects, and control methods for infectious diseases [1,2]. The agriculture and livestock sector is of critical importance in both economic and social terms. Viral infectious diseases in animals pose significant challenges to this sector. The economic impacts of viral infectious diseases include mortality rates, reduced product quality, treatment costs and mandatory measures such as culling [3]. It is particularly important to closely monitor diseases such as anthrax, bluetongue and avian influenza. In their study, Govindaraj et al. [4] detailed the financial costs of outbreaks on farms, demonstrating that mass culling measures-such as those implemented during avian influenza outbreaks-result in significant financial losses for farm owners. Bovine viral diarrhoea (BVD), on the other hand, suppresses the immune system of cattle, leading to secondary infections. The study by Walz et al. [5] comprehensively examined the spread of BVD and

its effects on health. Therefore, epidemiological analyses of such diseases are important for developing preventive and therapeutic strategies.

Interest in the dynamics of infectious diseases dates back a long way. The first known mathematical model was used by the renowned mathematician Bernoulli in 1760 to analyse a smallpox outbreak [6]. In the years that followed, the collection of statistical data on epidemic diseases led to studies covering different time periods and geographical regions, as well as the development of empirical mathematical models [7]. The control of viral diseases involves four stages: identifying risk factors, developing vaccination strategies, establishing early warning systems, and modeling disease spread. Initially, at-risk animals and regions are identified. Next, vaccination timing and target populations are defined, with control measures activated upon outbreak signs. Finally, intervention plans are created by predicting disease spread through epidemiological models. Mathematical and statistical models are essential in understanding disease transmission and shaping public health strategies [8]. They analyze the spread mechanisms, with models like SIR predicting outbreak progression and



identifying key parameters such as the basic reproduction number (R_0).

The aim of this study is to provide a basic introduction to the dynamics of the SIR (Susceptible, Infected, Recovered/Removed) mathematical model, the most commonly used model for modelling infectious diseases, and to illustrate the course of the disease through a simulation using transmission and recovery rates.

MATERIAL AND METHODS

Ethical Statement

This study did not involve any experimental procedures on animals or humans. The analyses were based on open-access data and simulation-based modelling; therefore, approval from an ethics committee was not required.

Study Material and Simulation Design

In this study, a simulation-based modelling approach based on the Susceptible-Infected-Recovered (SIR) epidemic model was used to predict the course of viral infectious diseases and recovery dynamics in livestock populations. The model was developed to estimate the number of animals expected to recover within a 10-day period in a herd of 1000 animals under different transmission and recovery rate scenarios.

The dynamics of the disease within the herd were analyzed by considering various combinations of infection transmission rates (β) and recovery rates (γ) specified by the researcher. Using these parameters, temporal changes in the numbers of susceptible, infected, and recovered animals were estimated. Analyses were conducted on different epidemiological scenarios created by varying the β and γ parameters to examine their effects on the disease transmission process. The basic reproduction number was calculated as $R_0 = \beta / \gamma$, and the potential for an outbreak was assessed.

Within the scope of the analysis, two main scenarios were examined over a 10-day simulation period. In the first scenario, while the infection transmission rate β was fixed at 0.004, recovery rates of $\gamma = 0.1$ and $\gamma = 0.5$ were evaluated. In the second scenario, the infection transmission rate was set at $\beta = 0.007$, and the recovery rate was taken as $\gamma = 0.3$. For each scenario, the expected numbers of susceptible, infected, and recovered animals were calculated over the simulation period and presented graphically to assess the progression of the disease and recovery patterns within the herd.

Software and Numerical Analysis

All simulations and numerical solutions were carried out using the “deSolve” package in the R programming environment. This package was used to solve the system of

differential equations forming the basis of the SIR model and to generate the relevant epidemic curves. By adjusting the selected model parameters, the proposed framework enables the prediction of disease transmission dynamics and recovery processes for flocks of different sizes and under varying epidemiological conditions.

Model Compartments and Transition Structure

The SIR model divides the herd population into three epidemiological groups: susceptible animals (S), infected animals (I), and recovered animals (R). The sum of these three groups yields the total population constant N . Susceptible animals are animals that have not yet been exposed to the disease but are capable of becoming infected. Infected animals are those that are actively infected and capable of transmitting the disease, and recovered animals are those that have recovered from the disease and gained immunity.

The model mathematically defines the rates at which animals move between these three categories and is used to analyse the progression of an epidemic over time. The way in which individuals transition between states in the model depends on various factors. Transition from the susceptible state to the infected state requires transmission of the disease, and this transition depends on the rate and prevalence of transmission. Transition from the infected compartment to the recovered compartment depends on the recovery rate of the infection. These transitions are summarised in Fig. 1.

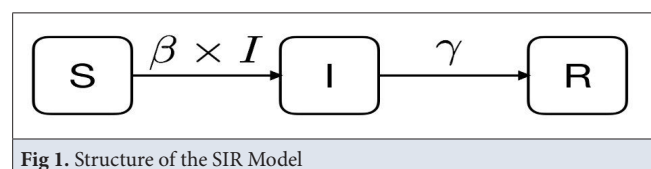


Fig 1. Structure of the SIR Model

Equations

Differential equations were used to mathematically define the SIR model. The equations represent the rate of change over time of the number of individuals in each compartment at any given time. The SIR model is defined by the following system of differential equations:

$$\frac{dS}{dt} = -\beta \frac{SI}{N}$$

$$\frac{dI}{dt} = \beta \frac{SI}{N} - \gamma I$$

$$\frac{dR}{dt} = \gamma I$$

In these equations, $N=S+I+R$ represents the total population size, β is the effective transmission coefficient, and γ is the recovery rate. In this formulation, the infection term is normalized by the total population size (N), allowing the transmission process to be represented independently of population magnitude and ensuring biologically realistic epidemic dynamics.

Transmission and Recovery Parameters

The model assumes that a transmission rate (p) applies to each contact. The value, generally referred to as the effective contact rate and denoted by β , is calculated as the product of the number of contacts (σ) and the transmission rate. Accordingly, the transmission rate, defined as the number of susceptible individuals moving from compartment S to compartment I during the time interval t , is expressed as:

$$\frac{\beta \times S \times I}{N}$$

where the parameter β represents the effective contact rate between susceptible and infected individuals. The recovery process is assumed to occur at a constant per capita rate γ , such that infected individuals move to the recovered compartment at a rate of:

$$\gamma \times I$$

where $\gamma = 1/D$, and D denotes the average infectious period [9].

Basic Reproduction Number

The basic reproduction number is an important measure of the strength of an infectious disease outbreak (R_0). In the normalized SIR framework, the basic reproduction number is commonly defined as:

$$R_0 = \frac{\beta}{\gamma}$$

which represents the average number of secondary infections generated by a single infected individual in a fully susceptible population. It is measured by counting the number of secondary cases following the introduction of the infection into a fully susceptible population [10]. In other words, it is the number of new infectious animals that an infected animal will produce during its infectious period when introduced into a fully susceptible herd. If $R_0 > 1$ in the population, the conditions are conducive to an outbreak, as each primary infection leads to more than one secondary infection. Conversely, if $R_0 < 1$, as an average primary infection does not lead to an additional secondary infection, the conditions for an outbreak to emerge do not arise. The level of herd immunity can

be measured by the magnitude of the decrease in the R value [11].

Statistical Analysis

The expected numbers of susceptible, infected, and recovered animals were calculated for each parameter combination over the 10-day simulation period. The effects of different infection transmission and recovery rates on epidemic progression were evaluated by comparing the temporal changes in the SIR compartments across the selected scenarios. The results were presented graphically to assess disease progression and recovery patterns within the herd.

RESULTS

Examples of SIR model applications in an epidemic can be given as follows.

For a herd of 1000 head over a 10-day period, with a contact rate (β) of 0.004 and a recovery rate (γ) of 0.5, the numbers

Table 1. Contact rate (β) = 0.004, recovery rate gamma (γ) = 0.5 for a herd of 1000 animals over a 10-day period, showing the number of suspected, infected and recovered animals in relation to the epidemic situation

Day	Susceptible	Infected	Recovered
0	999.0000000	1.00000	0.000000
1	963.7055761	31.79830	4.496125
2	461.5687749	441.91575	96.515480
3	46.1563480	569.50418	384.339476
4	7.0358807	373.49831	619.465807
5	2.1489407	230.12934	767.721720
6	1.0390927	140.41085	858.550058
7	0.6674074	85.44479	913.887801
8	0.5098627	51.94498	947.545162
9	0.4328913	31.56515	968.001960
10	0.3919173	19.17668	980.431400

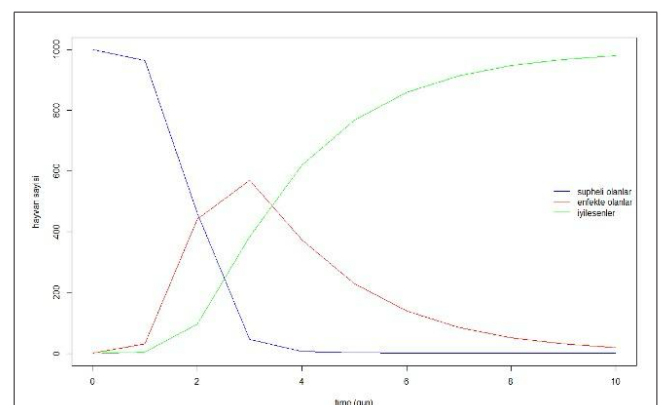


Fig 2. Graphical illustration of the SIR model with $\beta = 0.004$ and $\gamma = 0.5$ parameters

of suspected, infected and recovered animals during the outbreak are given in [Table 1](#) and shown in [Fig. 2](#).

Here, the basic reproduction number (R_0) is calculated as $\beta/\gamma \times 1000$ (N) = 8. The number of infected animals in the herd peaked at 569 on the third day following the onset of the outbreak, then declined, falling to 19 by the tenth day, whilst the number of recovered animals rose to 980.

The results of calculating the number of suspected, infected and recovered animals over a 10-day period for a herd of 1000 head, assuming a contact rate (β) of 0.007 and a recovery rate (γ) of 0.3, are presented in [Table 2](#), and the values shown in [Fig. 3](#) were obtained.

Table 2. Contact rate (β) = 0.007, recovery rate gamma (γ) = 0.3. Results when calculating the number of suspected, infected and recovered animals over a 10-day period for a herd of 1000 head in relation to an outbreak situation

Day	Susceptible	Infected	Recovered
0	9.990000e+02	1.00000	0.00000
1	5.447443e+02	429.26553	25.99019
2	2.947830e+00	747.37983	249.67234
3	3.163976e-02	555.96434	444.00402
4	1.096517e-03	411.89289	588.10601
5	9.078179e-05	305.13857	694.86134
6	1.434115e-05	226.05226	773.94773
7	3.653873e-06	167.46361	832.53638
8	1.362408e-06	124.06015	875.93985
9	6.179429e-07	91.90604	908.09396
10	3.523122e-07	68.08570	931.91430

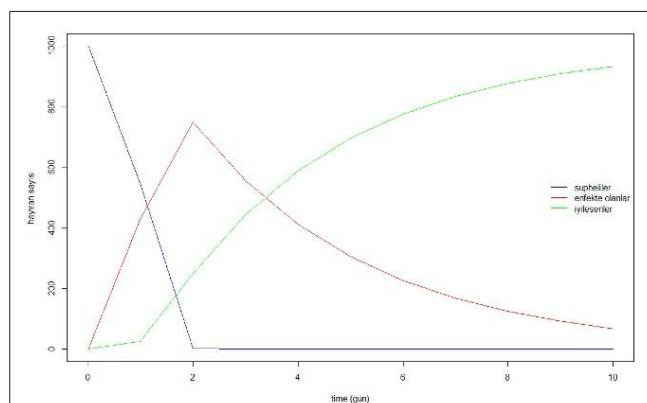


Fig 3. Graphical illustration of the SIR model with $\beta = 0.007$ and $\gamma = 0.3$ parameters

Here, the basic reproduction number (R_0) is calculated as $\beta/\gamma \times 1000 = 23$. As the basic reproduction number in the herd increased, the number of infected animals in the herd peaked at 747 on the second day; by the tenth day, this number had fallen to 68, whilst the number of recovered animals had risen to 931.

For a herd of 1.000 head over a 10-day period, with a contact rate (β) of 0.004 and a recovery rate (γ) of 0.1, the numbers of suspected, infected and recovered animals during the outbreak are presented in [Table 3](#), and the results shown in [Fig. 4](#) were obtained.

Table 3. Contact rate (β) = 0.004, recovery rate gamma (γ) = 0.1 for a herd of 1000 animals over a 10-day period, number of animals suspected, infected and recovered in relation to the epidemic situation

Day	Susceptible	Infected	Recovered
0	9.990000e+02	1.0000	0.000000
1	9.517764e+02	47.0130	1.210619
2	2.901416e+02	678.9487	30.909671
3	1.007621e+01	875.0093	114.914523
4	3.505072e-01	800.7713	198.878178
5	1.661487e-02	724.8784	275.105032
6	1.052368e-03	655.9116	344.087394
7	8.667749e-05	593.4942	406.505696
8	9.087673e-06	537.0159	462.984137
9	1.163745e-06	485.9121	514.087924
10	1.811918e-07	439.6715	560.328496

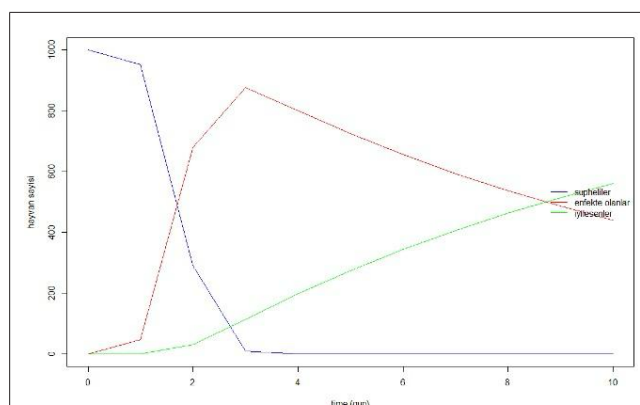


Fig 4. Graphical illustration of the SIR model with $\beta = 0.004$ and $\gamma = 0.10$ parameters

Here, the basic reproduction number (R_0) is calculated as $\beta/\gamma \times 1000 = 40$. In a herd where the contact rate is 0.004 but the recovery rate is as low as 0.1, 47 animals were infected on the first day, rising to 678 on the second day; by the tenth day, the number of infected animals remained at 439, whilst the number of recovered animals was limited to 560.

A summary of the course of the disease on the tenth day, based on three different simulation rates, is presented in [Table 4](#).

DISCUSSION

This study evaluated the role of the SIR model in the epidemiological analysis of infectious diseases within

Table 4. Disease progression on the 10th day based on varying contact rates, recovery rates, and basic reproduction numbers

Simulation	Contact Rate (β)	Recovery Rate (γ)	Reproduction Number (R_0)	Susceptible	Infected	Recovered
1	0.004	0.5	8	0.39	19.17	980.43
2	0.007	0.3	23	0.001	68.08	931.91
3	0.004	0.1	40	0.001	439.67	560.32

the framework of seroprevalence data on animal diseases in Türkiye and the broader mathematical modelling literature.

Examination of seroprevalence data concerning viral diseases in Türkiye reveals that these infections are widespread, yet exhibit pronounced regional variation. In a study encompassing 26 dairy cattle farms across different regions, BVDV seropositivity rates were found to range from 0.6% to 70.0% [12]. In the provinces of Ankara, Çorum, Kırıkkale, and Yozgat, this rate was determined to be 70.89% [13]. Similarly, IBR (Infectious Bovine Rhinotracheitis) seroprevalence demonstrated substantial variability, with seropositivity rates ranging from 14% to 81% [14]; in the North-Eastern Anatolia Region this figure ranged from 44.04% to 76.47% [15], while in the South-Eastern Anatolia Region it varied between 6.06% and 79.35% [16]. A comparable pattern was observed for bluetongue disease: seropositivity rates of 2.5% in Amasya and 10% in Sinop were detected in the Central Black Sea Region, whereas no seropositivity was identified in the provinces of Samsun, Ordu, and Giresun [17]. Seropositivity rates reached 65.2% in goats in Tokat and Samsun, while this rate was measured at 8.6% in sheep in Sivas [18]. These findings collectively demonstrate that the infections in question are widespread throughout Türkiye, yet seroprevalence varies considerably depending on geographical region, animal species, and local conditions.

These regional differences underscore the fact that disease dynamics cannot be addressed within a uniform framework, and that epidemiological models must be calibrated using local data. Indeed, herd health is critically important not only for protecting individual animal health, but also for preventing the spread of diseases at the population level [13,18]. In this context, control strategies -particularly vaccination- must be evaluated not merely from a clinical perspective, but also in terms of their epidemiological and economic dimensions [5,12].

Mathematical models have emerged as indispensable tools for understanding disease transmission mechanisms and designing control strategies. Basic epidemiological approaches such as the SIR model predict the course of an outbreak over time by examining the transitions of animals between different disease states within a population [10,19]. The basic reproduction number (R_0),

a parameter that occupies a central role in determining the critical dynamics of an outbreak, is widely calculated using the SIR model [8]. The principal factors influencing R_0 include the ease with which the virus spreads among animals via shared resources such as air, water, and feed; the incubation period of the disease; the duration during which infected animals shed the virus; herd density and contact frequency; the age and immune status of animals; and temperature, humidity, and hygiene conditions [9,20]. The accuracy and reliability of the predictions yielded by the model are, moreover, largely contingent upon the known transmission rate and seroprevalence data of the disease [21,22].

Recent studies have clearly demonstrated that the SIR model is not confined to providing a theoretical framework, but is equally capable of generating practical policy outputs. Zhang et al. [23], employing the SIR model in an integrated epidemiological-economic model designed to control a PRRS outbreak in Vietnam, determined an R_0 value of 1.3 and identified that the optimal vaccination rate should be approximately 26%, further reporting that the societal benefit of vaccination programmes was 2.3-4.5 times higher than the associated costs. These findings substantiate the capacity of SIR models to produce concrete economic outputs for livestock policy planning. Valdes-Donoso and Jarvis [24], in their analysis of intra-farm and inter-farm PRRS-transmission using an SIR model, demonstrated that vaccination programmes yield considerable economic benefits for both individual farms and regional production systems, placing particular emphasis on the decisive role of herd density, animal movements, and the distance between farms in transmission dynamics. In a study on Newcastle Disease Virus (NDV), Annapragada et al. [25], using an extended version of the classical SIR model to examine the effects of vaccination coverage on herd immunity, found that vaccination rates constitute a critical factor for the sustainability of poultry flocks, particularly in rural areas, and stated that a vaccination coverage of at least 40% is required to achieve a significant increase in flock population. These findings further highlight the importance of accounting for the dynamic nature of animal populations within mathematical models.

A review of the historical record reveals that epidemics have left deep impressions on societies and have given rise to serious public health problems [6]. This reality further

reinforces the critical role of mathematical and statistical modelling in understanding, controlling, and preventing infectious diseases [7,8]. Modelling approaches provide valuable insights into disease spread, its impacts, and control strategies [10]; by simulating in advance the effects of interventions such as vaccination, quarantine, and social distancing, they enable the identification of the most effective measures [21,22]; and they serve as an indispensable instrument for planning healthcare system capacity. It is also noteworthy that whilst statistical indicators such as disease incidence rates and recovery rates have found an established place of application in medicine, the insufficiency of such data in the livestock sector constitutes a conspicuous gap and signals an urgent need in this domain.

When all these findings are considered in their entirety, it becomes evident that SIR-based models can be effectively utilised not only in epidemiological analyses, but also in economic planning, the optimisation of vaccination strategies, and the formulation of biosecurity policies. It is understood that clinical approaches alone are insufficient for bringing infectious diseases under control, and that mathematical modelling methods must be integrated into decision-making processes [4,14]. The increasing mobility of animals and the consequent rise in the rate of viral disease transmission further underscore the importance of epidemiological models. Accordingly, the integrated use of SIR models with real-time field data, artificial intelligence-supported forecasting methods, and economic analyses will contribute substantially to the more effective management of outbreaks.

DECLARATIONS

Availability of Data and Materials: All simulations and numerical solutions were carried out using the “deSolve” package in the R programming environment.

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

Ethical Statement: The analyses were based on open-access data and simulation-based modelling; therefore, approval from an ethics committee was not required.

Declaration of Generative Artificial Intelligence (AI): The article and/or tables and figures were not written/created by AI and AI assisted technologies.

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RESEARCH ARTICLE

Chemical Composition, *In Vitro* Fermentability, and Anti-Methanogenic Potential of Oak (*Quercus* spp.) Leaves with PEG Supplementation

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Abstract

This study evaluated the chemical composition, *in vitro* fermentability, and anti-methanogenic potential of oak (*Quercus* spp.) (*Quercus brantii*, *Quercus cerris*, *Quercus coccifera*, *Quercus ithaburensis* subsp. *macrolepis*, *Quercus infectoria*, *Quercus libani*, and *Quercus suber*) leaves, focusing on condensed tannins and the effect of polyethylene glycol (PEG) treatment. Leaves from seven species were collected, dried, and analyzed for dry matter, crude protein, ether extract, crude ash, neutral detergent fiber, acid detergent fiber, and condensed tannins. Fermentation characteristics, methane production, metabolizable energy, and organic matter digestibility were assessed *in vitro*, with and without PEG. Chemical composition and tannin content varied significantly among species ($P<0.001$), with crude protein ranging from 10.25% to 17.41% and condensed tannins from 1.18% to 9.54%. PEG treatment significantly increased total gas production, methane production, metabolizable energy, and organic matter digestibility across all species ($P<0.001$), although methane percentage remained largely unaffected, suggesting tannins indirectly reduce methane rather than acting directly on methanogens. Among species, *Q. cerris* exhibited the highest gas production and digestibility after PEG, whereas *Q. brantii* showed the lowest fermentation efficiency. Species with higher tannin content displayed lower *in vitro* fermentability but greater methane suppression. Findings suggest oak leaves are a valuable feed resource that can enhance ruminant nutrition while helping control methane emissions. Utilizing tannin-rich leaves with PEG provides a dual benefit: improving feed efficiency and contributing to sustainable livestock management.

Keywords: *Quercus* spp., Oak leaves, Condensed tannins, Polyethylene glycol (PEG), methane, *In vitro* gas production

INTRODUCTION

Livestock production in ruminant animals is challenged by two main issues: maximizing animal productivity and reducing environmental impacts, including emissions of enteric methane, which are responsible for large amounts of greenhouse gases in the atmosphere^[1,2]. In this regard, tree leaves are seen as alternative or complementary feeds to traditional forages for ruminant animals because of their nutritional and bioactive properties. Of these, oak leaves are common and accessible in most parts of the world and are traditionally used as animal feed for ruminants because they provide them with protein, fiber, and mineral requirements for growth and maintenance^[3,4].

Although oak leaves are good for ruminant animals, their use is limited by secondary metabolites such as condensed tannins and phenolic compounds, which are anti-nutritional in nature and are responsible for reducing nutrient availability and feed efficiency in ruminants^[5,6]. Polyethylene glycol (PEG) is commonly used to reduce

tannins and phenolic compounds in oak leaves. PEG binds tannins and phenolic compounds, thus eliminating their anti-nutritional activities and improving nutrient availability and feed efficiency in ruminants without affecting nutrient value^[7,8].

Besides their effect on feed utilization, oak leaves also possess anti-methanogenic activity. Oak leaves are tannin-rich and are capable of reducing methane emissions in ruminants by inhibiting methanogenic archaea and reducing hydrogen availability, which is critical for methanogenesis^[6,9,10]. As oak leaves possess both activities, they are therefore promising for sustainable ruminant production systems.

There are significant differences in chemical composition and tannin and fermentation potential in oak leaves, depending on oak tree species, which affect their nutritional value and methanogenic activity^[3]. The interaction between oak leaves, PEG supplementation, and microbial activity in ruminants is critical in order to fully



understand and optimize oak leaves for ruminant feeding. This study aims to assess the chemical composition, *in vitro* fermentability, and anti-methanogenic activity of *Quercus* species (*Quercus brantii*, *Quercus cerris*, *Quercus coccifera*, *Quercus ithaburensis* subsp. *Macrolepis*, *Quercus infectoria*, *Quercus libani*, and *Quercus suber*), including PEG supplementation, in order to understand and optimize ruminant feeding for sustainable ruminant production.

MATERIAL AND METHODS

Collection and Preparation of Oak Leaves

Leaves of seven different oak species, namely, *Quercus brantii*, *Quercus cerris*, *Quercus coccifera*, *Quercus ithaburensis* subsp. *Macrolepis*, *Quercus infectoria*, *Quercus libani*, and *Quercus suber*, were collected from Kahramanmaraş province on May 12, 2025, and immediately transported to the lab. The leaves were dried at room temperature until complete desiccation was achieved. The dried leaves were then ground through a 1 mm sieve and finally stored in nylon bags. The leaves were reserved for further analysis of chemical compositions as well as *in vitro* gas production, ensuring standardized conditions for all experimental procedures.

Chemical Composition Analyses

The dry matter content was obtained by drying the sample at 105°C for 24 h. The crude ash content was obtained by incinerating the sample at 550°C for 6 h using a muffle furnace. The ether extract was obtained by using a Soxhlet extractor with petroleum ether. The crude protein was obtained using the Kjeldahl method as specified by AOAC [11]. The neutral detergent fiber and acid detergent fiber contents were obtained using the procedures specified by Van Soest et al. [12]. The condensed tannins contents were obtained using the butanol-HCl assay as specified by Makkar [13].

Determination of Gas and Methane Production of Oak Leaves

The *in vitro* gas production technique was employed to assess gas and methane production. According to Menke et al. [14], fresh rumen liquor was collected from slaughtered animals (sheep) at a local abattoir. The fresh rumen liquor was filtered through four layers of cheesecloth under anaerobic conditions. Each sample, consisting of 0.2 g of oak leaf sample with or without PEG 6000 (1 g), was placed in individual 100 mL glass syringes. Thirty milliliters of filtered buffer solution containing rumen liquor was added to each syringe under anaerobic conditions. The glass syringes containing sample-free blanks were used to assess gas production due to buffer solution. Each sample was carried out in triplicate at 39°C for 24 h using a

thermostatically controlled incubator. After 24 h of *in vitro* fermentation, gas produced was collected using a plastic syringe. Methane present in the collected gas was detected using an infrared methane analyzer (Sensor Europe GmbH, Erkrath, Germany) [15]. The concentration of methane was expressed as a percentage or milliliters. Net gas production and methane production for each sample were calculated by subtracting the respective blank values.

The volume of gas produced and methane concentration was employed to calculate metabolizable energy (ME) and organic matter digestibility (OMD) using equations developed by Menke & Steingass [16].

$$\text{ME (MJ/kg DM)} = 2.2 + (0.137 \cdot \text{GP}) + (0.057 \cdot \text{CP}) + (0.002859 \cdot \text{EE}^2)$$

$$\text{OMD (\%)} = 16.49 + (0.9042 \cdot \text{GP}) + (0.492 \cdot \text{CP}) + (0.387 \cdot \text{CA})$$

GP: Gas production (ml) at 24 h incubation

CP: Crude protein (%)

EE: Ether Extract (%)

CA: Crude ash (%)

Statistical Analysis

Analysis of the data was done using SAS (2005). The effects of species, PEG supplementation, and their interaction on chemical composition and fermentation parameters were determined by two-way ANOVA. Tukey's multiple comparison tests were used to compare the means. Significance was considered at $P < 0.05$.

RESULTS

The effect of species on the chemical composition of oak leaves was provided in [Table 1](#). The chemical composition of the leaves of seven *Quercus* species differed significantly ($P < 0.001$). The range of dry matter (DM) composition was from 32.94% for *Q. suber* to 51.27% for *Q. brantii*. The highest crude protein (CP) composition was found in *Q. suber* (17.41%), while the lowest CP composition was found in *Q. coccifera* (10.25%). The fiber composition of the leaves of different oak species varied. *Q. brantii* contained the highest composition of both NDF (51.15%) and ADF (33.82%), while *Q. suber* contained the lowest composition of NDF (36.45%) and ADF (26.25%). Condensed tannin (CT) composition was highest in *Q. cerris* (9.54%), while the lowest in *Quercus ithaburensis* subsp. *macrolepis* (1.18%).

Effect of species and PEG supplementation on *in vitro* gas ([Fig. 1](#)), methane ([Fig. 2](#)), metabolizable energy (ME) ([Fig. 3](#)), and organic matter digestibility (OMD) ([Fig. 4](#)) of different oak leaves is shown in [Table 2](#). *In vitro* fermentation of the seven *Quercus* species was significantly affected by

Species	DM	CA	EE	CP	NDF	ADF	CT
<i>Q. branti</i>	51.27 ^a	4.11 ^{cd}	2.34 ^a	11.69 ^{cd}	51.15 ^a	33.82 ^a	1.52 ^e
<i>Q. cerris</i>	50.11 ^{ab}	2.99 ^c	2.21 ^{ab}	11.35 ^{cd}	47.97 ^{ab}	32.20 ^{ab}	9.54 ^a
<i>Q. coccifera</i>	43.04 ^c	5.01 ^b	1.97 ^{ab}	10.25 ^d	43.40 ^{cd}	29.81 ^c	5.53 ^b
<i>Q. ithaburensis</i> subsp. <i>macrolepis</i>	48.68 ^b	3.42 ^{de}	1.74 ^{ab}	11.31 ^{cd}	46.60 ^{bc}	30.47 ^{bc}	1.18 ^e
<i>Q. infectoria</i>	49.25 ^b	6.33 ^a	1.87 ^{ab}	12.81 ^c	42.77 ^d	26.86 ^{de}	1.70 ^e
<i>Q. libani</i>	44.29 ^c	4.48 ^{bc}	1.51 ^{ab}	15.29 ^b	41.69 ^d	28.68 ^{cd}	3.84 ^c
<i>Q. suber</i>	32.94 ^d	4.79 ^{bc}	1.38 ^b	17.41 ^a	36.45 ^e	26.25 ^e	2.96 ^d
SEM	0.576	0.201	0.249	0.492	0.964	0.682	0.243
P	***	***	***	***	***	***	***

^{a,b,c,d,e} Column means with common superscript do not differ at $P < 0.05$, **DM**: Dry matter, **CA**: Crude ash, **EE**: Ether extract, **CP**: Crude protein, **NDF**: Neutral detergent fiber, **ADF**: Acid detergent fiber, **CT**: Condensed tannin, *** $P < 0.001$

Examples	GAS (mL)		Methane (mL)		Methane (%)		ME (MJ/kg DM)		OMD (%)	
	PEG		PEG		PEG		PEG		PEG	
	-	+	-	+	-	+	-	+	-	+
<i>Q. brantii</i>	19.56 ^b	29.46 ^b	2.30	3.80 ^b	11.60 ^{ab}	12.90 ^{ab}	5.53 ^b	6.90 ^b	40.73 ^b	49.63 ^b
<i>Q. cerris</i>	26.16 ^a	39.00 ^a	2.30	5.73 ^a	8.83 ^b	14.66 ^a	6.43 ^a	8.20 ^a	46.33 ^a	57.90 ^a
<i>Q. coccifera</i>	24.70 ^{ab}	29.10 ^b	2.70	3.5 ^b	10.93 ^{ab}	12.10 ^{ab}	6.16 ^{ab}	6.73 ^b	44.80 ^{ab}	48.80 ^b
<i>Q. ithaburensis</i> subsp. <i>macrolepis</i>	26.90 ^a	34.60 ^{ab}	3.23	4.13 ^b	11.93 ^a	11.83 ^b	6.53 ^a	7.56 ^{ab}	47.00 ^a	54.00 ^{ab}
<i>Q. infectoria</i>	23.23 ^{ab}	32.02 ^{ab}	2.46	3.46 ^b	10.60 ^{ab}	10.73 ^b	6.06 ^{ab}	7.26 ^{ab}	44.96 ^{ab}	52.90 ^{ab}
<i>Q. libani</i>	24.70 ^{ab}	30.93 ^b	2.86	3.80 ^b	11.40 ^{ab}	12.30 ^{ab}	6.43 ^a	7.30 ^{ab}	47.20 ^a	52.80 ^{ab}
<i>Q. suber</i>	26.16 ^a	32.76 ^{ab}	2.60	4.00 ^b	9.83 ^{ab}	12.13 ^{ab}	6.76 ^a	7.60 ^{ab}	49.60 ^a	55.56 ^{ab}
SEM	1.674	2.208	0.319	0.435	0.812	0.798	0.227	0.300	1.510	1.985
P	0.010	0.007	0.106	0.002	0.023	0.009	0.002	0.005	0.002	0.006
Species	***		***		NS		***		***	
PEG	***		***		***		***		***	
Species x PEG	NS		NS		***		NS		NS	

^{a,b} Column means with common superscript do not differ at $P < 0.05$, **ME**: Metabolizable energy (MJ/kg DM), **OMD** (%), **SEM**: Standard error mean, **PEG**: Polyethylene glycol, **NS**: Not significant, +: With PEG added, -: Without PEG added, *** $P < 0.001$

PEG supplementation ($P < 0.001$). The total gas production and methane production were significantly enhanced in all the tested species after PEG supplementation ($P < 0.01$). Among the tested species, *Q. cerris* had the highest gas production (26.16 mL without PEG, 39.00 mL with PEG) and methane production (2.30 mL without PEG, 5.73 mL with PEG), while *Q. brantii* had the lowest gas production (19.56 mL without PEG, 29.46 mL with PEG) and OMD (40.73% without PEG, 49.63% with PEG). Methane proportion (%) was not significantly affected by PEG supplementation ($P > 0.05$). The metabolizable energy (ME) and OMD were significantly enhanced by PEG supplementation in all the tested species ($P < 0.01$). The highest ME and OMD were observed in *Q. cerris* and *Q.*

suber after PEG supplementation. Significant differences were observed among the tested species in gas production, methane production (mL), ME, and OMD, while the interaction between species and PEG supplementation was significant only in methane proportion (%), suggesting that the relative response of methane production to PEG varies among species.

DISCUSSION

The present study revealed a strong species-specific variation with respect to the chemical composition as well as the *in vitro* fermentability of the leaves of **Quercus**, which largely accounted for the variations observed

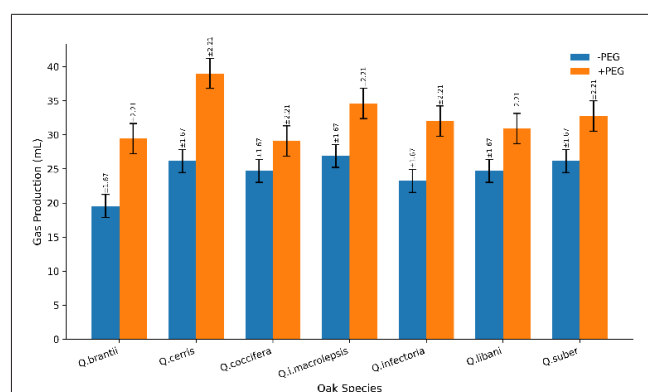


Fig 1. Effect of PEG addition on gas production

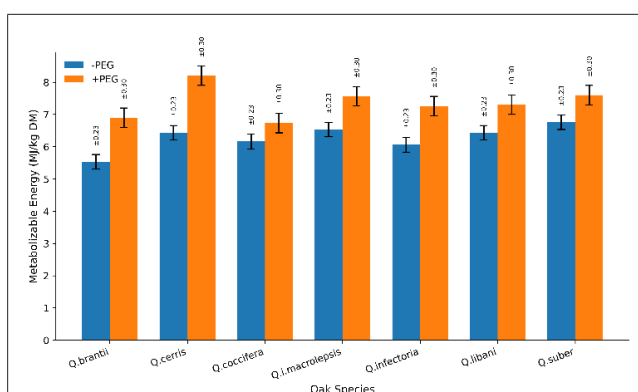


Fig 3. Effect of PEG supplementation on metabolic energy content

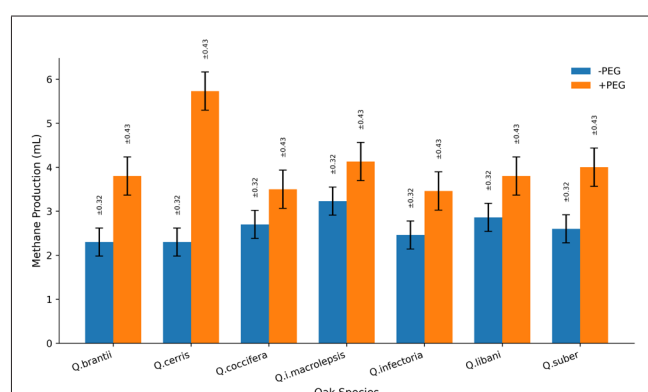


Fig 2. Effect of PEG addition on methane production (mL)

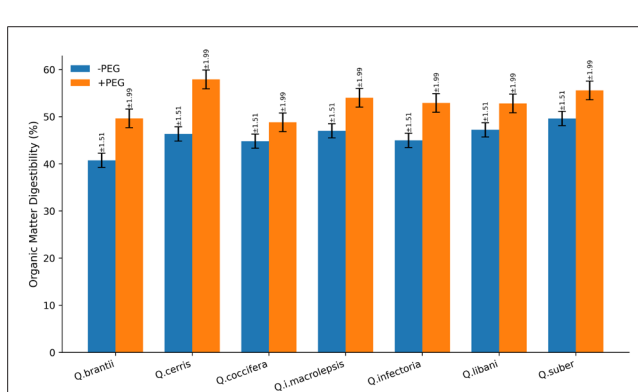


Fig 4. Effect of PEG supplementation on the degree of organic matter digestibility

with respect to the ruminal fermentation characteristics. Species like **Q. suber** and **Q. libani**, which possessed a higher crude protein content as well as a lower fiber content, showed a higher digestibility as well as energy availability, as previously established with respect to the nutritive value of oak leaves [3]. On the contrary, species with a higher content of condensed tannins, like **Q. cerris**, revealed a reduced fermentability but a higher potential for the suppression of methane production. This result is consistent with the well-established trade-off between the nutritive value and anti-methanogenic properties of tannin-containing feeds, as previously established [5,6].

The inclusion of polyethylene glycol significantly increased gas production, methane yield, metabolizable energy, and organic matter digestibility of all the species, validating the biological activity of tannins present in oak leaves. PEG effectively complexes with tannins, thus neutralizing their inhibitory effect on the activity of microbe-derived enzymes and substrate degradation [5,7]. This consequently allows rumen microflora to have greater access to available substrates for degradation, hence increasing the rates of fermentation. These results are consistent with other studies that have shown an increase in gas production and digestibility of feedstuffs supplemented with PEG in tannin-containing diets (Fig 1) [8,17,19,20].

Though there was an increase in the production of total methane (mL), the percentage of methane production (%) remained unaffected which indicates that tannins do not have a direct inhibitory effect on methanogenic microorganisms but have an indirect inhibitory effect by reducing the production of methane through the suppression of the overall process of microbial fermentation (Fig 2). This has been supported by earlier studies, which have suggested that the anti-methanogenic effects of tannins are related more to the reduced availability of hydrogen and the suppression of microorganisms as a whole, rather than the direct toxicity of tannins towards methanogens [5,9,18,21,22].

Most importantly, the failure to establish a clear relationship between CT concentration and fermentation parameters suggests that the effects of tannins cannot be accounted for by their concentration. The biological activity of tannins depends on their chemical structure, such as molecular weight, degree of polymerization, and protein binding affinity [5,6]. Consequently, it can be suggested that tannins at similar concentrations may have significantly different effects on rumen microbe fermentation. This may account for the variation observed between species.

Other compositional factors, aside from tannins, may have played a role in the observed variability, which include fiber composition and the potential involvement of secondary metabolites beyond tannins. The variability in NDF and ADF composition may affect degradation, while the structural composition, which may include lignin and other phenolic compounds, may have additional effects on microbial processes [9,18]. The above studies indicate that the responses to oak leaves in ruminal fermentation processes are likely the result of complex interactions among nutrient composition, tannin characteristics, and other plant secondary metabolites.

The positive effects of ME and OMD after PEG supplementation have again emphasized the negative effect of tannins on the nutritive values of feed (Fig. 3, Fig. 4) [23-25]. Tannins are known to have negative effects on digestibility by binding proteins and inhibiting fibrolytic activity [26-28]. This negative effect on digestibility is closely related to the positive effect of tannins on mitigating methane emission. From a practical point of view, the species that have greater effects on inhibiting fermentation may have greater mitigation effects on methane emission, while the species that have lower effects on tannins may have greater nutritive values.

Overall, the findings support the notion that tannins in oak leaves have a twofold impact on ruminant animals, reducing methane production while simultaneously reducing animal feed utilization. The twofold impact highlights the need to develop strategic feeding models in which tannin-containing forages or tannin-binding agents like PEG have the potential to be optimized in terms of animal production and environmental sustainability [1,2,23-26].

The present findings are consistent with previous reports indicating that condensed tannins exert complex and dose-dependent effects on rumen fermentation dynamics. Tannins are known to suppress methanogenesis primarily through indirect inhibition of methanogenic archaea and protozoal populations, as well as by decreasing fiber degradation and hydrogen availability in the rumen. This mechanism may explain the observed reduction in methane production associated with oak leaf inclusion. Similar reductions in enteric methane emissions have been reported in studies evaluating tannin-rich tree leaves and shrub species, suggesting that tannin-containing feed resources could contribute to environmentally sustainable ruminant production systems. However, the beneficial environmental effects of tannins are often accompanied by negative nutritional consequences. The reduced feed utilization observed in the present study may be attributed to the strong binding affinity of tannins to dietary proteins, carbohydrates, and digestive enzymes, resulting in lower nutrient digestibility and microbial activity. Excessive

tannin concentrations can particularly impair cellulolytic bacteria, thereby reducing fiber degradation and overall ruminal fermentation efficiency. Consequently, although methane mitigation represents an important advantage, decreases in nutrient utilization and animal performance may limit the practical applicability of high-tannin forages when used without dietary balancing strategies [9,18,29,30].

Nonetheless, as the present findings were conducted under *in vitro* conditions, caution is required when extrapolating the findings to *in vivo* systems. The response of animals may vary depending on certain parameters such as feed intake, adaptation of rumen microbiota, and the composition of the diet. Further *in vivo* research is required to determine the optimal inclusion levels, to assess the performance of the animals, and to confirm the methane mitigating potential under practical feeding conditions.

In conclusion the oak leaves are indicative of a promising natural feed source with co-benefits in providing protein to ruminants with moderate protein content while offering potential benefits in mitigating enteric methane production with its biologically active condensed tannins. The *in vitro* PEG assay confirmed that tannins in oak leaves are active in inhibiting rumen microbial fermentation because they increased gas and methane production in PEG-treated samples. This confirmed that the potential benefits of oak leaves in mitigating methane production are more dependent on the biological activity and chemical structure of tannins rather than their concentration. Oak leaves are promising as a sustainable and locally available feed source for mitigating enteric methane production in ruminants, but its inclusion in ruminant diets needs to be carefully optimized to meet protein protection, digestibility, palatability, and fermentation efficiency. Therefore, oak leaves are promising in providing substantial potential benefits as a feed source in mitigating enteric methane production in ruminants while promoting sustainable productivity.

DECLARATIONS

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CASE REPORT

Inflammatory Polyp of the Tympanic Cavity After Total Ear Canal Ablation with Lateral Bulla Osteotomy in a Dog

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Abstract

A 12-year-old dog presented with neurologic signs three years after bilateral total ear canal ablation with lateral bulla osteotomy (TECA-LBO). Based on clinical history, diagnostic imaging, and cytologic findings, cholesteatoma was suspected. Medical management was ineffective, and aggressive surgical excision was performed despite neurologic risk. However, histopathology confirmed an inflammatory polyp. This case highlights that inflammatory polyps may occur within the tympanic cavity following TECA-LBO in dogs and should be included among the differential diagnoses for tympanic cavity mass-like lesions, with consideration of this possibility during surgical planning.

Keywords: Dog, Total ear canal ablation, Lateral bulla osteotomy, Tympanic cavity, Inflammatory polyp

INTRODUCTION

Inflammatory polyps are non-neoplastic proliferative lesions arising from the epithelium of the middle ear, auditory tube, or nasopharynx, and are well recognized in cats but rare in dogs ^[1]. These polyps may extend into the external ear canal or nasopharynx and can cause clinical signs associated with local inflammation or mass effect ^[2]. Their etiology is unknown but may be associated with chronic inflammation, congenital malformations, and infection ^[2,3].

Total ear canal ablation with lateral bulla osteotomy (TECA-LBO) is widely performed as a salvage procedure for end-stage otitis externa or external ear canal masses ^[4]. Reported postoperative complications include facial nerve paralysis, Horner's syndrome, persistent discharge, para-aural abscess, fistula, and cholesteatoma ^[4,5]. This report describes a dog that developed an inflammatory polyp within the tympanic cavity three years after bilateral TECA-LBO. In dogs presenting with a mass-like lesion within the tympanic cavity after TECA-LBO, inflammatory polyps should also be considered among the differential

diagnoses, in addition to cholesteatoma. Consideration of this possibility during diagnostic imaging, cytologic evaluation, and surgical planning may help guide clinical decision-making in similar cases.

CASE HISTORY

Written informed consent was obtained from the owner prior to inclusion of the animal in this case report.

A 12-year-old female spayed Pomeranian with underlying diabetes mellitus initially presented to the internal medicine department of our hospital for episodes of generalized convulsions and nystagmus. The neurologic signs temporarily improved after dextrose supplementation at a local veterinary clinic but recurred, prompting referral to our hospital. On initial presentation, the patient showed a right-sided head tilt, bilateral vertical nystagmus, and vestibular ataxia. Vital signs were unremarkable. Serum chemistry revealed severely decreased glucose (9 mg/dL; reference interval [RI]: 65-118) and a mildly increased alkaline phosphatase (167 U/L; RI: 29-97). Blood gas analysis demonstrated a mild decrease in potassium (3.75 mmol/L; RI: 3.9-5.1). All other blood test results



were within normal limits. The marked hypoglycemia was considered the primary cause of the neurological signs and was suspected to be iatrogenic, likely related to inadequate insulin dose adjustment during diabetes management. The patient was hospitalized for blood glucose stabilization and further management, during which horizontal nystagmus was also observed.

Despite blood glucose stabilization, the vestibular signs persisted, making hypoglycemia an unlikely sole cause. Therefore, magnetic resonance imaging (MRI) was performed to further investigate the underlying cause of the neurologic signs (Fig. 1). A markedly expanded right tympanic cavity containing a rim-enhancing lesion with diffusion restriction was identified on MRI, a finding that had not been detected on physical examination. The left tympanic cavity showed only mild expansion with non-enhancing material. Brain parenchyma appeared normal. The patient had undergone bilateral TECA-LBO at a local veterinary clinic three years previously for chronic otitis externa, and therefore the external ear canals were absent on imaging. Computed tomography (CT) further confirmed a contrast-enhancing soft-tissue mass occupying the right tympanic cavity with bony destruction, while the left tympanic cavity contained non-enhancing soft-tissue material (Fig. 2). Based on the imaging findings, the neurologic signs were considered to be caused by the right tympanic cavity mass. The relevance of the left-sided

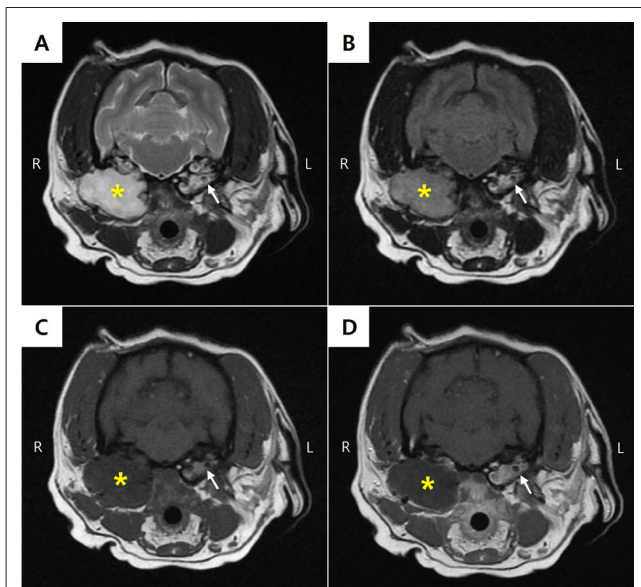


Fig 1. Magnetic resonance images of transverse T2-weighted (T2W) (A), FLAIR (B), and pre-contrast and post-contrast T1-weighted (T1W) sequences (C, D). The right tympanic cavity (yellow asterisks) is markedly expanded and filled with material that appears hyperintense on T2W, iso- to hyperintense on FLAIR, and hypointense on T1W, demonstrating peripheral rim enhancement on postcontrast T1W. The left tympanic cavity (white arrows) is mildly expanded and contains material that appears hyperintense on T2W, hyperintense on FLAIR, and hyperintense on T1W images, without appreciable contrast enhancement

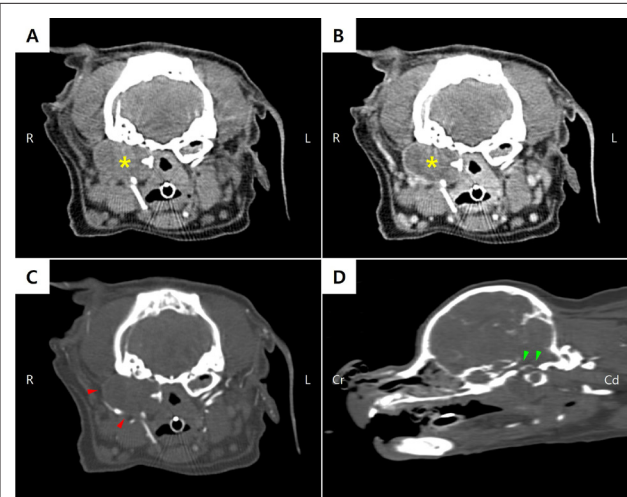


Fig 2. Transverse precontrast (A) and postcontrast (B) CT images in a soft tissue window, and precontrast CT images in a bone window in transverse (C) and sagittal (D) planes. The right tympanic cavity contains a space-occupying, expansile soft-tissue mass (yellow asterisks) that shows weak internal contrast enhancement with peripheral rim enhancement (A, B). The ventrolateral bulla wall is absent (C, red arrowheads). Osteolysis extending to the petrous temporal bone and the ventral trigeminal canal is observed (D, green arrowheads). The left tympanic cavity contains soft-tissue attenuation material without appreciable contrast enhancement

lesion was uncertain. Fine-needle aspiration identified keratinized epithelial cells and cocci, with keratin debris accounting for approximately 20% of the sample, and bacterial culture yielding positive. Antibiotic susceptibility testing demonstrated susceptibility to minocycline, and antimicrobial therapy was adjusted accordingly. However, clinical signs did not improve despite antimicrobial therapy. Therefore, surgical intervention was considered necessary to remove the underlying cause, and the patient was referred to the surgical service. Resection of the right tympanic cavity mass was subsequently performed.

Premedication was performed with midazolam (0.1 mg/kg IV), and anesthesia was induced with propofol (3 mg/kg IV) and maintained with isoflurane in oxygen. Intraoperative analgesia was provided by remifentanyl administered as a constant rate infusion (CRI; 0-8 µg/kg/h, titrated to effect) throughout surgery.

With the patient in left lateral recumbency, a skin incision was made over the right tympanic cavity, and the subcutaneous tissues were bluntly dissected. A capsulated mass was identified and dissected using sterile cotton swabs and monopolar electrocautery. The medial aspect of the mass was adherent to the bone and lacked a distinct capsule. The mass was incised to obtain samples for bacterial culture and antimicrobial susceptibility testing, and the adherent portion was removed (Fig. 3). Intraoperative bleeding was controlled with compression and a hemostatic powder. The cavity was irrigated, inflammatory debris was aspirated, and a drainage tube

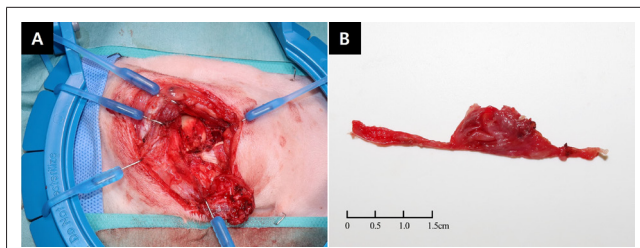


Fig 3. Intraoperative findings and gross appearance of the inflammatory polyp. (A) Lonestar retractors were applied to optimize surgical exposure, allowing identification of an inflammatory polyp occupying the right tympanic cavity. (B) The excised inflammatory polyp, which had been incised intraoperatively for culture sampling, exhibited an irregular surface owing to its detachment from the adjacent bony structures

constructed from a 19-gauge butterfly catheter and a plain tube was placed to provide active suction. The resected mass was fixed in 10% neutral buffered formalin and submitted for histopathologic evaluation (IDEXX Laboratories, Inc., USA). Examination revealed an inflammatory polyp with pleocellular otitis media, lined by stratified squamous epithelium (*Fig. 4*).

Postoperative recovery from anesthesia was uneventful. Postoperative analgesia consisted of a continuous remifentanyl infusion during the initial postoperative period, followed by transition to a fentanyl transdermal patch (1.66 µg/kg/h), with the patient discharged on postoperative day 3. The drain produced approximately 1 mL of fluid within the first 3 hours after surgery and was subsequently removed as no further drainage was observed. The surgical site healed without complications, and the suture removal was performed 14 days after surgery. Based on the results of bacterial culture and antimicrobial susceptibility testing, the postoperative antibiotic was changed to ciprofloxacin in consultation with the internal medicine team, initiated 1 week after surgery and continued for one month. Facial nerve paralysis was noted immediately after surgery, characterized by right-

sided lagophthalmos, loss of the palpebral reflex and drooling. Right-sided head tilt persisted postoperatively, and bilateral horizontal nystagmus was intermittently observed with excitement. The patient received ocular supportive care with hyaluronic acid eye drops and lubricant ointment, but corneal edema developed 20 days postoperatively, for which a bandage contact lens was applied. With continued ocular management, the cornea remained healthy, although facial nerve dysfunction persisted throughout the 5-month follow-up period. The owner was informed of the need for continued ocular care and has been compliant with the recommended management.

DISCUSSION

Incomplete removal of epithelial remnants during TECA-LBO may allow these cells to persist within the tympanic cavity and, under chronic inflammation, proliferate and lead to complications including persistent infection, para-aural fistula formation or cholesteatoma [5-7]. In the present case, the patient underwent bilateral TECA-LBO three years previously for chronic otitis externa, and a soft tissue lesion was identified within the right tympanic cavity. These findings, together with the absence of brain parenchymal abnormalities on MRI, suggest that the observed vestibular signs were most likely associated with peripheral vestibular dysfunction secondary to the tympanic cavity lesion [8,9]. Based on previous reports of cholesteatoma as a postoperative complication of TECA-LBO [6], the lesion was presumed to be a cholesteatoma and surgical intervention was performed. However, histopathologic examination confirmed an inflammatory polyp lined by stratified squamous epithelium. This finding indicates that inflammatory polyps can occur within the tympanic cavity after TECA-LBO and may mimic cholesteatoma clinically.

Inflammatory polyps and cholesteatomas are non-neoplastic mass lesions that may arise within the canine middle ear [1,5,10]. Inflammatory polyps can be treated by traction-avulsion (TA) or TECA-LBO, and some authors advocate a conservative surgical approach, with TA as an initial treatment and TECA-LBO reserved for recurrent cases [1]. Cholesteatomas are characterized by the accumulation of keratinizing stratified squamous epithelium within the tympanic cavity, resulting in progressive bulla enlargement, osteolysis, and potential involvement of adjacent neural structures with subsequent neurologic dysfunction [11]. Consequently, aggressive surgical excision is recommended, which may require thorough curettage and removal of affected bone using rongeurs or a high-speed burr [5,6]. Inflammatory polyps have a relatively low recurrence rate of approximately 8%, whereas recurrence rates of up to 50% have been

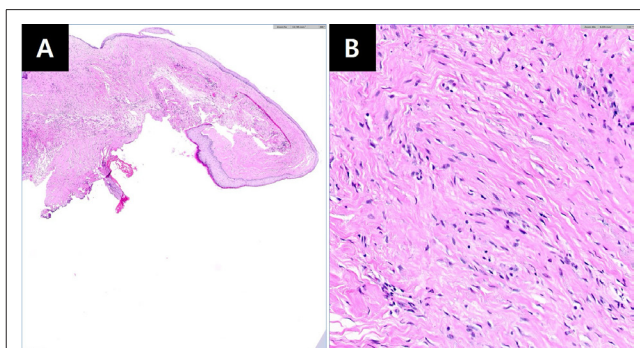


Fig 4. Histopathologic features of the inflammatory polyp. (A) The polyp is lined by well differentiated keratinizing stratified squamous epithelium, and mixed inflammatory infiltrates consistent with pleocellular otitis media are present (Hematoxylin and eosin stain, Magnification $\times 50$, Scale bar 200µm). (B) The collagenous stroma of the polyp is observed (Hematoxylin and eosin stain, Magnification $\times 400$, Scale bar 50 µm)

reported for cholesteatomas ^[1,6]. Therefore, preoperative differentiation of middle ear masses is critical for surgical planning and prognosis, and imaging and cytologic findings should be carefully considered.

On CT imaging, both lesions may appear as soft-tissue attenuation masses with tympanic bulla osteolysis, making differentiation challenging ^[3,12]. However, contrast-enhancement patterns differ, with inflammatory polyps typically showing intralesional enhancement whereas cholesteatomas composed of avascular keratin debris demonstrate peripheral rim enhancement without intralesional enhancement ^[3,12]. Reports describing MRI features of inflammatory polyps are limited, and the MRI characteristics of cholesteatomas are not yet well established ^[1,3,13]. Although cytological evaluation may be non-specific, as mixed inflammatory debris, bacteria, and keratinizing squamous epithelial cells can be observed in both lesions, recent literature suggests that a cytological diagnosis of cholesteatoma is supported only when keratin debris comprises 80-100% of the sample ^[3,6,13]. Application of these criteria to the present case allows an interpretation different from the preoperative presumptive diagnosis.

Beyond these two non-neoplastic lesions, neoplasia should also be considered in the differential diagnosis of a mass-like lesion within the tympanic cavity ^[14]. Primary middle ear tumors are extremely rare in dogs, and squamous cell carcinoma is the most commonly reported tumor type ^[10,14]. In addition, neoplasia may extend medially from residual external ear canal epithelium ^[14]. A recent CT-based study reported that intralesional mineralization, intralesional fluid/debris, and regional lymphadenopathy were common CT findings in canine ear canal tumors ^[10]. Cytologic evaluation may also provide supportive evidence for neoplasia when neoplastic cells are identified ^[14,15]. However, none of these CT findings were identified in the present case, and cytology did not reveal neoplastic cells, suggesting that neoplasia may be less likely.

In the present case, the intralesional enhancement on CT and the low proportion of keratin debris on cytology indicated an inflammatory polyp should have been strongly considered as a differential diagnosis ^[3,13].

Nevertheless, because cholesteatoma has been described as a postoperative complication of TECA-LBO ^[6], the lesion was presumed to be a cholesteatoma and surgery was performed. Consequently, more aggressive dissection and excision were performed ^[5,6], resulting in postoperative facial nerve paralysis. Had an inflammatory polyp been included in the preoperative differential diagnosis, a more conservative initial excision followed by histopathologic evaluation, with subsequent staged surgical decision-making, might have been considered.

This case suggests that inflammatory polyps should be considered an important differential diagnosis in addition to cholesteatoma in dogs presenting with a mass-like lesion within the tympanic cavity after TECA-LBO. In particular, when intralesional contrast enhancement is observed on CT and the proportion of keratin debris on cytology is low, a surgical approach that accounts for the possibility of an inflammatory polyp may be considered to help reduce the risk of complications associated with aggressive excision. Furthermore, these findings underscore the importance of thorough removal of epithelial remnants during TECA-LBO. Overall, awareness that inflammatory polyps may occur within the tympanic cavity after TECA-LBO may provide practical guidance for clinicians when making surgical and therapeutic decisions in similar cases.

DECLARATIONS

Availability of Data and Materials: The datasets analyzed during the study are available from the corresponding author (S. Lee) on request.

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Authors' Contributions: Conceptualization was done by SJ, YK and SL, data curation was done by SJ and YK, visualization was done by SJ and YK, supervision was done by SL, and the article was written by SJ, YK and SL.

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• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

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ARTICLE EVALUATION AND PUBLICATION PROCESS**• 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.

• Preliminary Evaluation Process

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 subject 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.

• Peer-review Process

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• Publication Process of an Article

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

Processing and publication are free of charge with the journal. There is no article processing charges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

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

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.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Üniversitesi Veteriner Fakültesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Üniversitesi Veteriner Fakültesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Üniversitesi Veteriner Fakültesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Üniversitesi Veteriner Fakültesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (E-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

2- The official language of our journal is English.

3- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and other files (figure etc.) should be submitted by using online manuscript submission system at the address of <http://vetdergi.kafkas.edu.tr/>

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

4- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that “informed consent” was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

5- Authors should know and take into account the “Generative Artificial Intelligence (AI)” and other matters listed in the “**Ethical Principles and Publication Policy**” section regarding scientific research and authors.

6- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

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.

“Invited review” articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

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

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

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.

8- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

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.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: McIlwraith CW: Disease of joints, tendons, ligaments, and related structures. **In**, Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

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

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as “et al” and “and friends” should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;

<https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university>

9- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

10- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

11- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checked by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

12- The editorship may request the language editing of the manuscript submitted to the journal. If the article is accepted, it will not be published without language editing. Before publication, a declaration and/or certificate stating that proofreading is done by a registered company will be requested from the corresponding author.

13- No fee is charged at any stage in Kafkas Üniversitesi Veteriner Fakültesi Dergisi (No APC/APF)

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover Letter

- Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).
- Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere).

- Title Page

- Title, Running Title (should be a brief version of the title of your paper, no exceed 50 characters)
- The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)
- Congress-symposium, project, thesis etc. information of the manuscript (if any)
- Corresponding author's address, phone, fax, and e-mail information

- Manuscript

- Title, abstract, keywords and main text
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided

- Declarations

- Availability of Data and Materials
- Acknowledgements
- Funding Support
- Competing Interests
- Generative Artificial Intelligence (AI)
- Authors' Contributions

Further Considerations

- Journal policies detailed in this guide have been reviewed
- The manuscript has been "spell checked" and "grammar checked"
- Relevant declarations of interest have been made
- Statement of Author Contributions added to the text
- Acknowledgment and conflicts of interest statement provided

