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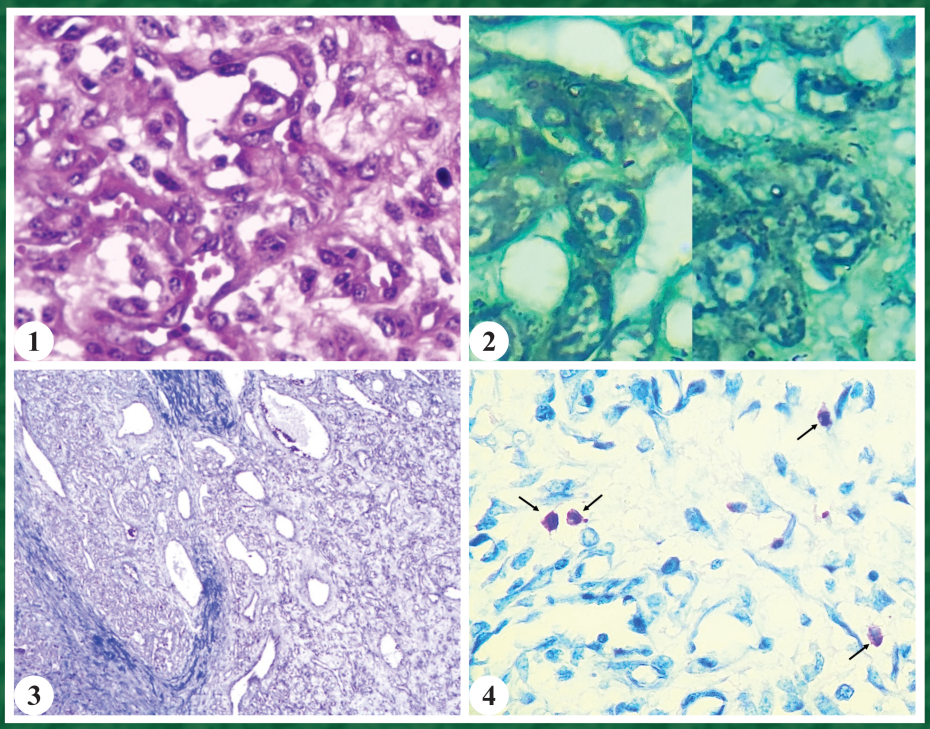

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Cover Page Photo (Clockwise) : Capillary haemangioma in a calf: Neoplastic capillaries are lined by single layer of plumpy endothelial cells (H&E x400) (Left top), Stippled chromatin (resembling salt and pepper) of proliferating vascular endothelial cells (Heidenhain's hematoxylin with light green counter stain x1000) (Right top), Neoplastic mass shows separation of lobules with collagen bundles (Masson trichrome stain x40) (Left bottom) and Scattered mast cells (arrow) in stromal tissue of capillary haemangioma (Standard toluidine blue stain x400) (Right bottom).

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REVIEW ARTICLE

Elephant endotheliotropic herpesvirus haemorrhagic disease of Asian elephants: An updated mini review

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ABSTRACT

Asian elephants *(Elephas maximus)*, the National Heritage Animal of India and classified as endangered by the IUCN. Their existence is threatened by habitat loss, poaching, deforestation and emerging diseases such as elephant endotheliotropic herpesvirus (EEHV), which has become a major cause of mortality among elephant calves over the past three decades. First reported in North America in 1995, EEHV has since caused severe losses in both captive and wild populations throughout the world. In India, the first case was recorded in Kerala in 2013. The virus is mainly transmitted through direct mucosal contact, trunk secretions or fomites and may also spread *via* saliva or intestinal contents. Latent nature of EEHV permits to establish a carrier status in adult Asian elephants and intermittent shedding of the virus without associated clinical disease. EEHV infection classically targets endothelial cells, resulting in widespread haemorrhage, DIC and cardiovascular failure. Clinical signs range from lethargy and facial oedema to acute death. Diagnosis is primarily based on nucleic acid detection methods like both conventional PCR and qPCR methods. The serological method like ELISA is developed to assess antibody status. Despite numerous attempts, EEHV could not be isolated in the cell culture system to date. Therapeutic management includes anti-herpes viral drugs like famciclovir, ganciclovir or acyclovir combined with intensive supportive care. Recent research on EEHV vaccines (viral vector and mRNA vaccines) shows promising results. High fatality rate and widespread occurrence, enhanced surveillance, rapid diagnostic capabilities and development of effective vaccines are crucial for mitigating the impact of EEHV on the conservation of Asian elephants.

Keywords: Asian elephant, endotheliotropic, glycoprotein B, haemorrhage, India, latency

INTRODUCTION

India harbours a rich diversity of wildlife that plays a vital role in maintaining ecological balance and the stability of natural ecosystems. Wildlife plays a vital role in regulating populations, facilitating nutrient cycling and maintaining energy flow within food webs, thereby supporting overall ecosystem stability and environmental health[1]. However, increasing anthropogenic pressures such as habitat loss, fragmentation, pollution and climate change have made elephant more vulnerable to several health-related problems, including nutritional and physiological stress, exposure to toxic substances and infectious diseases[2]. Among India's diverse megafauna, elephants hold a place of profound ecological significance and enduring cultural and religious reverence. The Asian elephant *(Elephas maximus)*, belonging to the family *Elephantidae* under the order *Proboscidea*, is the largest living terrestrial mammal. India supports nearly 60% of the global wild Asian elephant population[3]. Despite this, the species is listed as endangered by the IUCN due to habitat degradation, poaching and disease threats[4,5]. Like other mammals, elephants are susceptible to several infectious diseases such as tuberculosis, haemorrhagic septicaemia, rabies, foot and mouth disease and parasitic infestations[6,7]. In recent years, Elephant Endotheliotropic Herpesvirus (EEHV) infection has emerged as one of the most serious diseases affecting elephant populations worldwide. EEHV was first identified in African elephants, and the earliest fatal case in Asian elephants was reported[8]. Several subtypes of EEHV have been identified, among which EEHV-1A and EEHV-1B are predominantly associated with fatal haemorrhagic disease in Asian elephants[9,10]. The disease, termed Elephant Endotheliotropic Herpesvirus Haemorrhagic Disease (EEHV-HD), is an acute and highly fatal condition that primarily affects calves and juvenile elephants under 15 years of age[11]. In India, the first confirmed EEHV fatality was reported in 2013, and subsequent studies have documented EEHV-HD in Indian elephant populations[12-14]. Over the past three decades, Asian elephants have faced a serious threat from EEHV infection, which has emerged as one of the leading causes of mortality among juvenile elephants worldwide, including in India.

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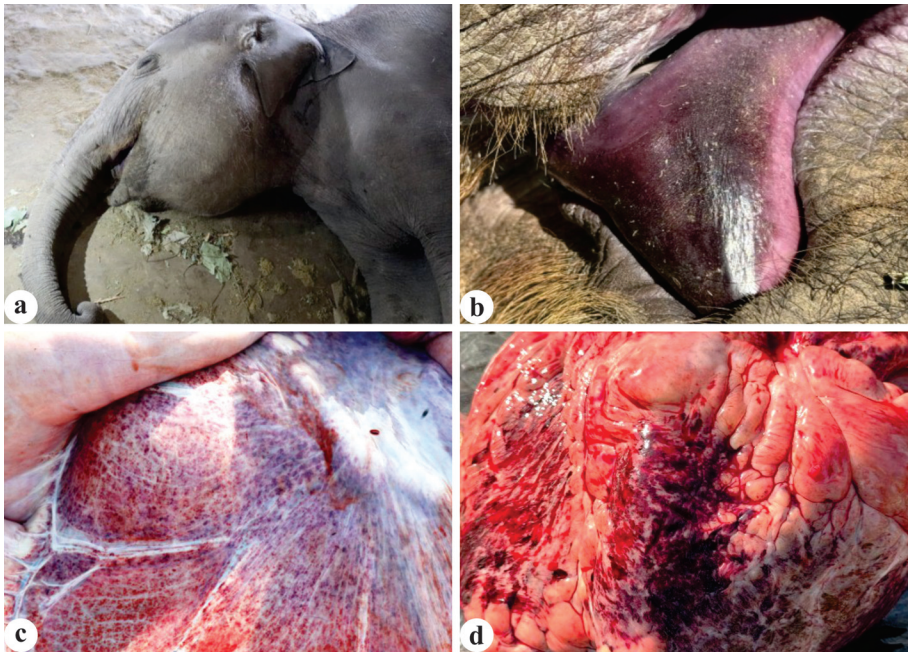
Elephant endotheliotropic herpes virus

The novel elephant herpes



Necropsy findings of EEHV-HD. a. Carcass of elephant calf showing marked swelling of the face due to severe subcutaneous oedema (Kalagarh Range, Jim Corbett National Park). **b.** Diffuse cyanosis of tongue. **c.** Diffusely congested mesenteric blood vessels with haemorrhages. **d.** Diffuse epicardial haemorrhages.

virus is currently classified within the genus Proboscivirus of the subfamily *Betaherpesvirinae*. However, recent genomic analyses of EEHV have led to the proposal of a new subfamily, *Deltaherpesvirinae*, to accommodate these viruses[15]. However, the proposed classification has not yet been officially adopted by the International Committee on Taxonomy of Viruses (ICTV). The EEHV cause a spectrum of infections in elephants, ranging from subclinical or localized lesions to acute, often fatal, haemorrhagic disease. The first lethal case of an acute haemorrhagic disease of unknown aetiology in an Asian elephant was reported from a circus in Switzerland in 1988[8]. Later, characteristic intranuclear inclusion bodies in vascular endothelial cells of a 16-month-old Asian elephant calf that died at the Smithsonian's National Zoo, USA confirming the involvement of a novel herpesvirus, subsequently named EEHV-1[9]. To date, several subtypes of Probosciviruses have been identified, including EEHV-1A, EEHV-1B, EEHV-2, EEHV-3, EEHV-4, EEHV-5, EEHV-6 and EEHV-7[10,16]. Asian elephants are primarily affected by EEHV-1, EEHV-4 and EEHV-5, which exhibit variable pathogenicity, whereas EEHV-2, EEHV-3, EEHV-6 and EEHV-7 have been identified in African elephants, with EEHV-2, EEHV-3 and EEHV-6 associated with fatal infections[10,16,17]. These viruses are believed to have co-evolved with their host species[18]. Although cross-species transmission from African to Asian elephants was once proposed, genomic analyses revealed that EEHV-1 is endemic to Asian elephants, while African elephants harbour different EEHV genotypes[10]. Genomic characterization has shown that EEHVs diverged independently from other mammalian herpesviruses millions of years ago[19]. The EEHV genome (-180 kb) encodes about 115-120 genes, including 35 conserved core genes and nearly 60 novel genes not found in other herpesviruses[10,15]. EEHV-1 and EEHV-2 form an A + T-rich branch (-42% GC), whereas EEHV-3 and EEHV-4 are G + C-rich. At the nucleotide level, EEHV-1 and EEHV-2 differ by -25%, while EEHV-1A and EEHV-1B show 15-40% variation in several glycoprotein genes[20]. Hypervariability in glycoprotein-H and vGPCR1 regions of EEHV-1 suggests long-term host-virus co-evolution[13]. Among the currently recognized Probosciviruses, EEHV-1 is the most pathogenic and prevalent[10]. The disease, termed EEHV-HD, is now recognized as a major cause of mortality in juvenile Asian elephants[11]. Calves between one and five years of age are most susceptible, while adults often remain asymptomatic carriers that intermittently shed the virus[21]. Protection in calves below one year of age is attributed to maternal antibodies[10]. Molecular genotyping of EEHV cases indicates that infections are sporadic rather than epidemic, with no evidence of inter-facility spread among captive herds[10,16].

Epidemiology of EEHV

The EEHV-HD has caused significant mortality in captive Asian elephants, with up to 65% of young, captive-born calves in Europe and North America succumbing to the disease[22]. The disease has also been reported across Asia, including India, Myanmar, Malaysia, Cambodia, Laos, Thailand, Nepal and Sumatra[11,17,23-27]. EEHV is primarily transmitted through direct contact with infected

bodily secretions, including saliva, trunk secretions and intestinal contents, with aerosol or droplet spread likely in close-contact situations[20,28]. Vertical transmission *via* placenta or breast milk is suspected but not fully understood. Latent infections can reactivate under stressors such as pregnancy, weaning or husbandry changes, leading to viral shedding without clinical signs. Pregnant elephants, particularly in the third trimester, show higher viral shedding[20,29] though shedding can occur independently of stress hormone levels.

Young, seronegative calves are most susceptible to EEHV-HD, with trained or recently weaned individuals disproportionately affected. EEHV-1 is associated with higher mortality than other subtypes. Cases occur in both captive and wild populations, across sexes and are more frequent during the rainy season[10,30]. Viral shedding is linked to pregnancy and elevated oxidative stress markers such as ROS and MDA[31]. Subclinical infections, documented in Asia, Europe and North America enable elephants to carry and disseminate EEHV without showing symptoms[32,33]. Latent infections allow the virus to persist in host cells, reactivating under stress or external stimuli[34]. Endothelial cells of umbilical cord vessels are suspected latency sites for EEHV-1.

Pathogenesis of EEHV-HD

The pathogenicity of EEHV depends on multiple host factors like[20,29]:

1. Age at exposure to the virus
2. Presence of passively transferred protective maternal antibodies obtained through nursing or during gestation
3. Presence of any immunocompromising disease states
4. Primary infection of EEHV is said to result in severe infection of EEHV, as most elephants found dead due to EEHV-HD were sero-negative for specific antibodies against EEHV
5. Young elephant calves are most susceptible to infection
6. Majority of positive cases are reported in trained or weaned calves
7. More number of cases are reported in rainy season
8. EEHV1 causes severe mortality in Asian elephants than other EEHV subtypes
9. Increased viral shedding is documented in pregnant animals

During the acute phase of infection, EEHV exhibits tropism for monocytes/macrophages, endothelial cells and epithelial cells of the elephant's alimentary tract, which serve as the primary sites for viral replication[35]. The virus mediated endothelial cell injury leads to increased vascular permeability and leakage with severe, widespread oedema and haemorrhage.

Virus entry and dissemination

In most herpesvirus, the binding of virion to host cell involves the interaction of Glycoprotein B (gB) with cell surface heparan sulphate proteoglycans and the virus enter either through fusion of the virion envelope with the plasma membrane or by endocytosis. The gB present in most herpesvirus is cleaved by cellular furin and such a conserved furin cleavage site is also found in EEHV-1 gB protein which helps to cleave the protein in the centre[36]. The specific cellular receptor for EEHV has not yet been identified.

A study on antibody production against EEHV demonstrated that salivary glands and gastrointestinal epithelial cells are the main target tissues for EEHV-1A and EEHV-4 infections. The presence of EEHV gB antigen in salivary glands has been confirmed through immunohistochemical analysis, suggesting that these glands may serve as the primary sites of viral replication for both EEHV-1A and EEHV-4[28]. The virus spreads throughout the elephant's body via infected blood monocytes[37]. EEHV gB antigens have been detected in PBMCs within the blood vessels of internal organs[28]. This pattern is similar to betaherpesviruses like cytomegalovirus, which replicate in salivary glands and spread through monocytes and macrophages[38].

Apoptosis of PBMCs is commonly seen in fatal EEHV-1A-HD cases[37]. EEHV viral particles present within circulating monocytes are transferred to endothelial cells of the small blood vessels through monocyte adhesion to specific molecules expressed on the endothelial surface[39]. The interactions between the leukocytes and endothelial cells are initiated by a variety of chemical mediators derived from inflamed tissues, and the entire process of adhesion is regulated by the sequential activation of different families of adhesion molecules expressed on endothelial cells and the surface of leukocytes. The expression of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), a leukocyte adhesion receptor and endothelial ligand, has been demonstrated by immunohistochemistry. PECAM-1-positive cells were found to be significantly more prominent in EEHV-HD-positive tissues compared to EEHV-negative controls, indicating their potential role in the pathogenesis of the disease[39].

Endothelial damage

Endothelium is considered as an endocrine organ which plays a major role in the maintenance of homeostasis in the body. The name of the virus itself suggests its endotheliotropic nature. EEHV shows strong tropism for endothelial cells of the tongue, heart and liver[40]. Intranuclear inclusion bodies indicate severe infection of endothelial cells in large myocardial arteries and the endocardium[41]. EEHV preferentially infects endothelial and smooth muscle cells of small

vessels such as arterioles, venules and capillaries. This may be due to unique surface antigens and lower blood pressure in small vessels, which favour viral adhesion and infection[39]. The exact mechanism of endothelial damage in EEHV-HD remains unclear, though viral-mediated injury is considered the most likely cause, with immune-mediated damage and endothelial apoptosis also possible contributors[10]. Infection of EEHV-1A/B. EEHV-2, EEHV-4 and EEHV-5 primarily affect capillary endothelium, whereas larger vessel changes were mostly seen in EEHV-3 affected elephants[42]. As affected organs number in EEHV positive cases is more, viral dissemination can potentially happen through circulating EEHV infected endothelial cells[41]. The endothelial cells may also act as sites of latency of EEHV virus like Human cytomegalovirus[41].

Although endothelial cells play an important role in EEHV pathogenesis, viral load found more in liver and heart, which is less vascular than lungs, suggest that hepatocytes or cardiac myocytes may also support *in vitro* replication of virus[41]. The elevated level of Cardiac troponin in high viraemic elephants indicates cardiomyocytes damage[43]. Viral DNA is widely distributed across organs, with the highest loads in the liver for EEHV-1A and in the heart for EEHV-1B and EEHV-5 fatal cases[41]. Another study reported higher viral loads in the heart and lower in the brain[44]. Immunohistochemistry using gB protein showed positive signals in salivary gland epithelium, PBMCs in the spleen and heart and lymph node follicles[28]. Increase in the vascular permeability results in leakage of intravascular fluid through the endothelial gaps and results in oedema. Intranuclear inclusion bodies detected in endothelium in all the EEHV-HD cases, which demonstrates direct viral damage to the endothelium[18].

Formation of thrombus and DIC

Endothelial injury and dysfunction play a key role in thrombus formation. Normal endothelium allows smooth blood flow, but when damaged, it exposes collagen and releases procoagulants like von Willebr and factor (vWF). vWF forms a bridge between collagen and platelet receptor GpIb, promoting clot formation. It is also a marker of endothelial dysfunction and is produced by both endothelial cells and megakaryocytes[45]. In EEHV-1A HD and EEHV-4 HD, vWF antigens are mainly detected in the heart, intestine and lungs[39]. Activated platelets release granules and produce thromboxane to enhance haemostasis. Excessive platelet uses leads to DIC, characterized by bleeding and microthrombi[46].

Thrombocytopenia in EEHV is due to platelet consumption rather than destruction or decreased production, as bone marrow megakaryocyte numbers remain unchanged[35]. Cyanosis of the tongue, considered a pathognomonic lesion of EEHV-HD, represents a late clinical manifestation of overt disseminated intravascular coagulation (DIC). The cyanotic appearance results from severe oedema and intramuscular haemorrhage within the lingual tissues[18]. However, because of the absence of predominant ischaemic lesions, thrombosis is not likely to be the primary cause of EEHV-HD coagulopathy[18]. The DIC and irreversible damage to blood vessels subsequently leads to failure of organs or hypovolemic shock followed by the death of the elephant[47]. Systemic inflammation in EEHV is also hypothesized due to increase in the proinflammatory cytokine mRNA expression and this results in destruction of smaller blood vessels and followed by disseminated intravascular coagulopathy[48]. Upregulation of cytokines like IL-1, IL-2, IL-4, IL-8 and TNF-α were observed in EEHVHD infected cases[39]. In addition to severe tissue damages produced by cytokines, it is also suggested that cytokines are also important in EEHV infection control in young elephants by mediating innate immune response[18]. Recent study, using RNAscope® *in situ* hybridization, EEHV-1A DNA polymerase and terminase genes were detected in multiple archival tissues, including the heart, lung, tongue, spleen, liver, kidney, lymph node, gastrointestinal tract, salivary gland and central nervous system. The virus was found to replicate exclusively within capillary endothelial cells, with the highest viral loads observed in the heart and liver, suggesting that tissue-specific endothelial heterogeneity contributes to variation in viral replication and lesion severity[49]. Furthermore, significantly elevated expression of IFN-γ, IL-6 and IL-10 in tissues with high viral loads indicates a cytokine storm-like response as a key pathogenic mechanism in EEHV-HD, underscoring the potential benefit of incorporating anti-inflammatory or immunomodulatory therapies in clinical management[50].

Pathomorphological alterations of EEHV-HD in Asian elephants

Clinical findings

EEHV-HD among Asian elephants develops in a rapid progression and results in death within 1-5 days[51]. The mortality rate in calves that exhibits clinical signs is about 85%[52]. In most cases the disease starts with general clinical signs like lethargy, anorexia and dullness[51,53]. Early clinical signs of EEHV-HD often begin with non-specific symptoms such as lethargy, fever, changes in sleep patterns, reduced appetite and water intake, colic and stiffness[10,18]. Affected elephants may show subcutaneous oedema of the head, neck and limbs, along with swelling of the temporal glands and scleral injection[40,53]. As the disease progresses, severe manifestations like generalized oedema, oral ulceration, tongue and tracheal cyanosis develop, often leading to death within a week[40,54]. Diarrhoea is a common clinical symptom observed in EEHV infections[51,53]. In addition to

these signs abdominal colic was also noticed in positive cases[44]. The reactivation of the virus in reservoir animal results in non-pathogenic clinical signs which includes ulceration and vesicle formation in oral and vaginal mucous membranes[11].

A consistent haematological hallmark of EEHV-HD is acquired thrombocytopenia, reflecting the disease's association with thrombosis and haemorrhage[53]. It represents the most prominent and diagnostically significant haematological alteration observed in infected elephants[39]. Other common findings include leukopenia, anaemia, dehydration and systemic inflammation characterized by heterophilia with left shifting and toxic leukocyte changes[11,39]. EEHV-HD calves typically show anaemia, thrombocytopenia, monocytopenia and reduced plasma protein levels[15]. EEHV viremia in juvenile elephants is characterized by mild to moderate thrombocytopenia and leukopenia and moderate to severe monocytopenia[55]. Elevated acute phase proteins such as serum amyloid A (SAA) and haptoglobin (HP) are also reported in EEHV-HD[33]. Their levels are significantly higher in viraemic elephants and correlate with viral load, while TNF-α and IL-2 show minimal variation between infected and non-infected animals[56].

Gross lesions

Prominent gross lesions include subepicardial and myocardial haemorrhages, cyanotic tongue, oedema and extensive petechial to ecchymotic haemorrhages across multiple organs, notably the heart, lungs, liver, mesentery and gastrointestinal tract[12,40,57]. Additional findings such as pericardial effusion, ascites, hepatomegaly, intestinal ulceration and oedema of the trunk, limbs and head are commonly observed[10,40]. Coinfections with EEHV subtypes (e.g., EEHV-1A and EEHV-4) produce similar lesions, with more severe cardiac haemorrhages noted in EEHV1A cases[28,41]. Gross gastrointestinal lesions may resemble bacterial infections such as *Clostridium perfringens* or *Salmonella* spp. and cardiopulmonary changes can mimic *Citrobacter freundii* infection, highlighting the need for differential diagnosis[55].

Microscopical findings

Histopathological lesions in fatal EEHV-HD cases are characterized by severe vascular damage and endothelial involvement. Typical findings include extensive extravascular haemorrhage, myocardial fibre separation and intranuclear inclusion bodies within capillary endothelial cells[40,53]. In some cases marked vascular and intestinal oedema, haemorrhages and inflammatory infiltration by heterophils and lymphocytes were reported, along with basophilic inclusions in hepatic endothelial cells[39,40].

Co-infections with EEHV-1 and EEHV-4 demonstrated large intranuclear inclusions in myocardial arteries and endocardium[28]. The lesions predominantly involve small blood vessels and arterioles, characterized by varying degrees of haemorrhages and oedema[40]. Endothelial cells of the tongue, liver and heart often display apoptosis, swelling and inclusion bodies, consistent with immunohistochemical detection of viral antigens in these sites and splenic macrophages[58]. Additional vascular changes such as necrosis, hypertrophy, fibrinoid degeneration and oedema are frequently observed, with viral particles confirmed in endothelial cells of the tongue, spleen and heart[42,51].

Diagnosis of EEHV-HD

Diagnosis of EEHV-HD can be performed using clinical signs, gross lesions and histopathological lesions. Immunohistochemical detection using antibody against pan-EEHV antigen or gB is also commonly used for the diagnosis purpose[28,39,40]. Culture and isolation of viruses are considered as the gold standard method of diagnosis of any viral infection. The attempts for culturing and propagating the EEHV in different cell culture systems like primary fibroblasts, white blood cells and placental epithelial cells and umbilical cord vascular endothelial from Asian and African elephants was carried out in various parts of world[36,41,51]. All these efforts to develop the EEHV in cell culture settings were ineffective. EEHV cultured in elephant endothelial cells obtained from Asian elephant umbilical cord survived up to eight passages[59]. Elephant fibroblast cell lines showed growth in four passages when inoculated with EEHV infected elephant tongue along with infected PBMC[36]. Viral particles are also detected in the endothelial cells of tongue, spleen and heart[42,51]. Electron microscopy revealed accumulation of non-enveloped viral particles within the endothelial cells of the liver, associated with perinuclear cytoplasmic electron-dense bodies in EEHV-positive cases[36].

Polymerase chain reaction (PCR) remains the most widely used method for the confirmatory diagnosis of EEHV infection. First detected EEHV DNA in tissues of affected elephants using conventional PCR in 1999[51]. Later subtype specific PCR was developed using universal primer targeting the polymerase gene locus for broad EEHV detection, followed by type-specific primers for genotyping, enabling sensitive and specific diagnosis[52]. However, conventional PCR cannot reliably detect latent infections with low viral loads[29]. To improve sensitivity a quantitative real-time PCR (qPCR) assay was developed for capable of detecting EEHV-1 DNA in blood and trunk wash samples from both symptomatic and asymptomatic elephants[20]. qPCR enables the detection of early viraemia in clinically asymptomatic elephants, thereby facilitating timely therapeutic intervention and potentially reducing mortality[20,40,51]. Although qPCR enables quantification, it cannot determine viral infectivity, as *in vitro* culture of EEHV remains unsuccessful. The highest EEHV-1 viral

loads in bone marrow, heart, liver and lymphoid tissues of fatal cases were reported[36]. Loop-mediated isothermal amplification (LAMP) is another rapid nucleic acid amplification method used for EEHV detection in blood. This assay targets the POL gene of EEHV-1 and offers a detection limit about 100-fold higher than conventional PCR[60]. Additionally, RNAscope® *in situ* hybridization (ISH) has been employed to confirm EEHV1A infection in formalin-fixed tissues[49]. Recently, targeted enrichment of EEHV using ultracentrifugation to concentrate viral copies for complete genome sequencing were attempted[61].

Even though no commercially available diagnostic kits are available for detection of EEHV, several inhouse diagnostic kits are developed like ELISA to detect IgG antibodies of EEHV in serum or plasma samples collected from 125 captive Asian elephants[62]. The antigen used for developing the ELISA is gB, an envelope protein present in herpes virus. Recently, another ELISA was developed using the non-structural protein DNApol with the aim to detect EEHV shedders[21]. Indirect immunofluorescence is also used to view the viral proteins expression like gB protein in infected cells[36]. A serological test developed to differentiate infections with different EEHV, is Luciferase immunoprecipitation system (LIPS)[63]. This method helps in determining whether EEHV infection associated illness and fatalities occurred due to primary infection or reactivation of the latent virus. This method is also employed for assessing the susceptibility of elephant calves to EEHV infections and monitoring immune reactions to anti-EEHV vaccines. More recently, a 10% sero-prevalence of EEHV-1 among captive Asian elephants in India using inhouse developed ELISA was observed in the randomly collected samples[64].

Treatment of EEHV-HD

Treatment of EEHV-HD is very challenging and mostly antiviral medications often used in human medicine to treat herpesvirus infections, with a poor and unreliable success rate are used in this disease. Herpesvirus infections can be treated using nucleoside analogues such as famciclovir, acyclovir and[65,66]. The commonly used drug for EEHV treatment is famciclovir, which requires oral or rectal route of administration and it was found to be ineffective in clinical cases due to the increase in the viral load even after the administration of the drug. Intravenous route of administration is more preferred for the drugs such as acyclovir and ganciclovir[65,66].

Numerous cases of non-survival have been reported among elephants treated with anti-herpes viral drugs; therefore, early detection followed by prompt symptomatic and supportive therapy is considered more effective than antiviral treatment alone. Loss of vascular integrity led to fluid loss and fluid therapy is recommended for EEHV treatment[65]. For counteracting secondary bacterial infection antibiotics and to reduce pain due to inflammation analgesics can also be used. Due to the cardiovascular damage, intranasal oxygen therapy is also suggested in EEHV-HD[65,66]. The successful treatment of an EEHV-1 infected elephant calf with Zelnate also documented[67].

Vaccination

The non-availability of vaccines is also a major problem facing the control of this disease. In recent years, vaccine development efforts have primarily focused on identifying immunogenic viral proteins and optimizing delivery systems capable of inducing robust protective immune responses. The major viral envelope glycoproteins gB, gH and gL have been recognized as key targets due to their roles in viral entry and their ability to elicit neutralizing antibodies. Recombinant protein-based vaccine trials using EEHV-1A gB and gH/gL antigens have shown promising induction of virus-specific humoral responses *in vitro* and in preliminary elephant studies[30,68]. Further advancements include a multivalent EEHV1A mRNA vaccine encoding glycoproteins gB, gH, gL and gO, which demonstrated safety and the ability to elicit strong humoral and T-cell immune responses in mice, representing an important preclinical step toward an effective EEHV vaccine[69]. Similarly, a recombinant EEHV-1A gB subunit vaccine containing gBF1 and gBF2 fragments formulated with Montanide™ ISA 206 VG or incomplete Freund's adjuvant induced strong humoral and $CD4^+$ T-cell (Th1 and Th2) responses in mice, suggesting the potential of gB-based vaccines for EEHV prevention[70]. Although significant progress has been made, no fully protective or commercially available EEHV vaccine exists yet, underscoring the need for continued research to define correlates of protection, optimize adjuvant systems and evaluate vaccine efficacy in elephants.

Major conservation challenges in the management of EEHV-HD in India

The major challenges in management of EEHV include :

1. High mortality in juvenile elephants
2. Lack of detailed knowledge on the prevalence of EEHV and its subtypes circulating among Asian elephants in India
3. Lack of routine screening and monitoring procedures
4. Lack of knowledge of disease among forest officials and veterinarians
5. Elephants are not trained for collection of samples for routine clinical examination
6. Lack of well-developed diagnostic infrastructures in captive facilities
7. No commercially available vaccines/diagnostic kits

8. Non availability of effective anti-viral drug for treatment

There is an urgent need of standard national guidelines for EEHV-HD management in India, as current practices vary significantly across states. Differences in diagnostic protocols**,** including sampling methods and qPCR testing capacity, result in inconsistent detection and delay in confirmation. Additionally, data sharing between states, zoos, research institutions and forest departments remains fragmented, limiting the development of a robust national database. As a result, a cohesive and comprehensive national EEHV management framework is still evolving, highlighting the need for unified guidelines and coordinated implementation across the country.

CONCLUSION

Asian elephants in India now face conservation challenges due to the frequent outbreaks of EEHV-HD, a disease that excessively affects young elephants. Although significant progress has been made globally in understanding the molecular characteristics and diversity of EEHV strains, research in India remains limited. Intensive and widespread screening is urgently required to determine the true burden of the disease in both wild and captive populations, along with the development of point care diagnostic tools capable of detecting infections at subclinical stages. The continued absence of vaccines or targeted antiviral therapies underscores the need for enhanced research efforts. To address these challenges, a coordinated national strategy including qPCR-based screening, regional diagnostic laboratories, treatment protocols and real time surveillance systems are essential. Ultimately, safeguarding India's endangered Asian elephants will require sustained scientific research, strengthened institutional collaboration especially involving Project Elephant, MoEF & CC and leading research institutes with quick, well integrated conservation actions.

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RESEARCH ARTICLE

Histopathological and Immunohistochemical Insights into Ovarian Tumours in Sheep: A Comprehensive Study

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ABSTRACT

Ovarian tumours, though common in domestic animals, have been sparsely studied in sheep due to their early slaughter upon infertility. This study investigated 212 ovaries collected from 106 sheep to identify and characterize neoplasms. Histopathological and immunohistochemical analyses revealed one sex cord-stromal neoplasm (granulosa cell tumor), three epithelial neoplasms (adenoma, adenocarcinoma, clear cell carcinoma) and four mesenchymal neoplasms (hemangiosarcoma, fibroma, angioleiomyoma, hemangiopericytoma). Granulosa cell tumours were the most prevalent and exhibited distinct histological patterns, including Call-Exner bodies and multifocal coalesced patterns. Adenocarcinoma and cystadenoma presented characteristic growth patterns, while mesenchymal neoplasms exhibited unique histological and immunohistochemical features, such as VEGF positivity in hemangiosarcoma and SMA positivity in angioleiomyoma. These findings align with previous reports in other species, emphasizing the importance of immunohistochemistry in tumor classification and contributing valuable insights into the occurrence and pathology of ovarian tumors in sheep.

Keywords: Histopathological evaluation, immunohistochemistry, ovarian neoplasms, sheep ovaries

INTRODUCTION

Ovarian health is paramount to the reproductive efficiency and overall productivity of domestic animals, directly impacting the profitability of livestock industries. Among the various reproductive disorders, ovarian tumours, though relatively less common than conditions like cystic ovaries, represent a significant pathological concern due to their potential to severely compromise fertility and fecundity across a wide range of species, including cattle, goats, sheep, dogs, cats and mares[1]. While the occurrence of such neoplastic conditions has been documented in veterinary literature, comprehensive and in-depth investigations specifically focusing on ovarian tumours in sheep remain notably limited[2].

Ovarian tumours are classified into three main categories based on their origin: epithelial tumours, sex cord-stromal tumours and mesenchymal tumours. Epithelial tumours, the most frequently observed type, typically arise from the surface epithelium of the ovary, with rare instances originating from the rete ovarii[3,4]. Sex cord-stromal tumours arise from the non-germ cell components of the ovarian stroma and sex cords and include granulosa cell tumours, luteomas, thecomas, Sertoli cell tumours, Leydig cell tumours and roblastomas, arrhenoblastomas and lipid cell tumours[3]. Mesenchymal tumours, though exceedingly rare, include fibromas, hemangiomas, leiomyomas and their malignant counterparts[3,5].

With recent advancements in veterinary pathology, immunohistochemistry (IHC) has become an indispensable tool for diagnosing ovarian tumours. IHC enables precise identification of cellular lineages, differentiation patterns and specific protein markers within neoplastic tissues[6]. These markers help distinguish between tumour subtypes that may appear similar on routine H&E staining and can also reveal the presence of poorly differentiated cells. In the present study, IHC was applied to ovarian samples that had been positively identified with neoplastic changes during histopathological evaluation. Monoclonal antibodies against key markers, including Vascular Endothelial Growth Factor (VEGF), Pan-Cytokeratin and Smooth Muscle Actin (SMA) were used to provide a detailed cellular and structural characterization of ovarian tumours in sheep.

This comprehensive study aims to address existing knowledge gaps regarding ovarian tumours in sheep by first conducting a through histopathological examination to classify these tumours based on their cellular origin and morphological characteristics. Subsequently, it investigates the expression patterns of specific

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immunohistochemical markers across different tumor types, thereby providing detailed cellular and structural insights.

MATERIALS AND METHODS

The present study focused on identifying various ovarian neoplasms in 212 ovaries collected from 106 sheep of different age groups, approximately ranging from two to seven years. The samples were obtained from slaughter-houses in and around Tirupati, necropsied animals at the Department of Veterinary Pathology, College of Veterinary Science, Tirupati, and field mortality cases.

Table 1. Occurrence (%) of neoplastic conditions in the sheep ovaries.

S. No.	Name of the neoplastic condition	Number of cases	Occurrence (%)
	Sex cord neoplasm		
1.	Granulosa cell tumour	16	41.03
	Epithelial neoplasms		
2.	Cystadenoma	10	25.64
3.	Adenocarcinoma	2	5.12
4.	Clear cell carcinoma	2	5.12
	Mesenchymal neoplasms		
5.	Hemangiosarcoma	4	10.25
6.	Fibroma	2	5.12
7.	Angioleiomyoma	2	5.12
8.	Hemangiopericytoma	1	2.56
	Total	**39**	**100**

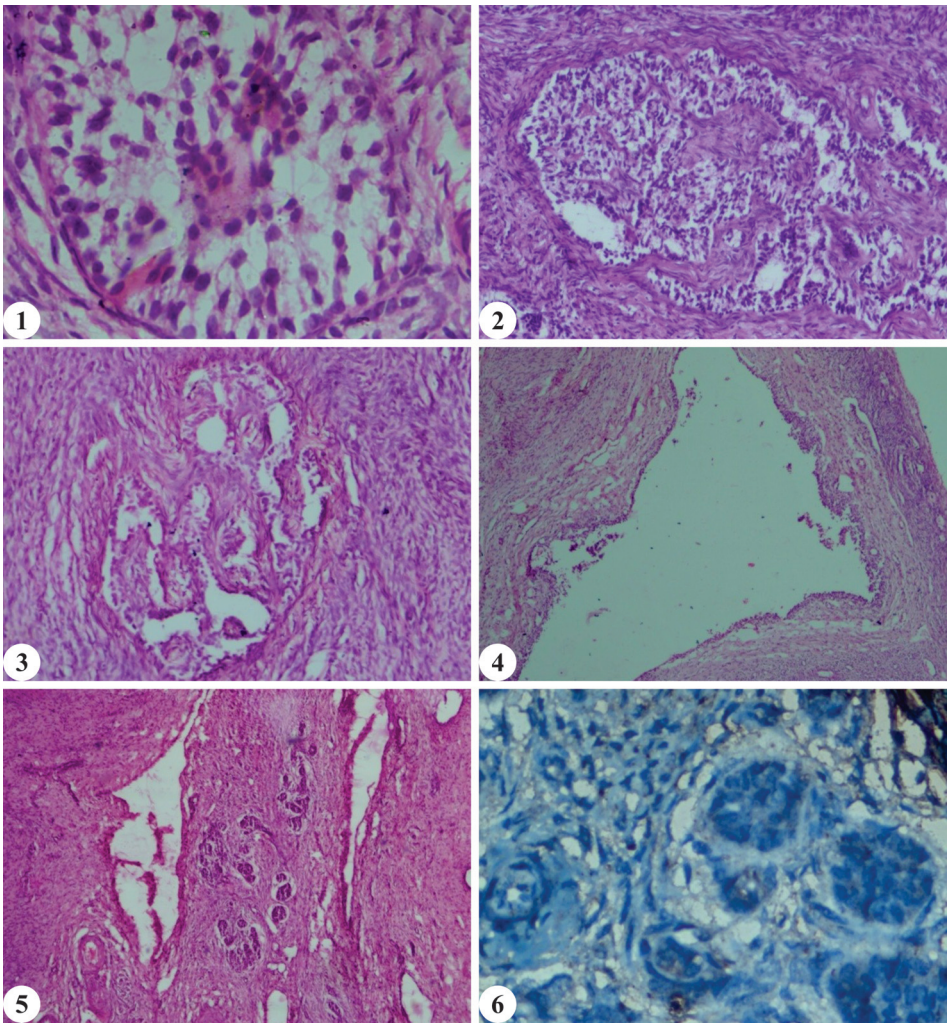

Fig. 1. Granulosa cell tumour: Neoplastic granulosa cells forming Call-Exner bodies, arranged in a distinctive rosette pattern around eosinophilic material (H&E x400); **Fig. 2.** Multifocal coalesced granulosa cell tumour: The infiltrated fibrous tissue made the neoplastic area into a multifocal coalesced pattern (H&E x100); **Fig. 3.** Cystadenoma of the ovarian stroma: Neoplastic epithelial cells projecting into the cystic space are supported by collagenous fibers (H&E x100); **Fig. 4.** Cystadenoma of rete ovarii: Irregular cystic space near the hilus surrounded by a thick layer of proliferated fibrous tissue, with the lining epithelium desquamated into the lumen (H&E x400); **Fig. 5.** Adenocarcinoma: Neoplastic epithelial cells arranged in a tubulo-acinar pattern in the ovarian stroma and supported by fibrous stroma (H&E x40); **Fig. 6.** Adenocarcinoma: Pan cytokeratin revealed mild cytoplasmic positivity within the neoplastic cells, observed as a brown color (IHC x400).

Necropsies were performed according to standard protocols[7]. For histological analysis, the ovaries were first subjected to a gross examination and then fixed in 10% neutral buffered formalin. The tissues were processed through a graded alcohol series, cleared with xylene, embedded in paraffin and cut into 4-5 µm thick sections using a Leica manual rotary microtome. These sections were then deparaffinized, rehydrated through decreasing alcohol concentrations and stained with hematoxylin and eosin (H&E) following standard procedures[8].

For IHC duplicate paraffin sections of the ovary were cut at 3-4 µm thickness, mounted on 3-Aminopropyltriethoxysilane (APES) coated slides, and incubated overnight at 37^{O}C. Sections were deparaffinized in xylene (two changes, 15 minutes each), rehydrated through graded alcohols and washed in distilled water. Antigen retrieval was performed using the heat-induced method with a pressure cooker (three whistles) in Tris-EDTA buffer (pH 9.0) and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Sections were washed in Tris buffer (pH 7.6) and incubated with primary antibodies (VEGF, Pan Cytokeratin and SMA) for 30 minutes at room temperature (25±5). After washing in Tris-EDTA buffer, super enhancer solution was applied for 20 minutes, followed by a secondary antibody with poly-horseradish peroxidase (Poly-HRP) for 20 minutes. Diaminobenzidine (DAB) was used as the chromogen and sections were counterstained with Harris hematoxylin, blued in lithium carbonate and mounted in DPX (kits from Bio Genex, USA).

RESULTS

The study identified eight ovarian neoplasms: one sex cord-stromal neoplasm (granulosa cell tumoUr), three epithelial neoplasms (adenoma, adenocarcinoma and clear cell carcinoma) and four mesenchymal neoplasms (hemangiosarcoma, fibroma, angioleiomyoma and hemangiopericytoma) (Table 1). Granulosa cell tumour was the most prevalent, followed by adenoma and hemangiosarcoma. While no gross lesions were characteristic, histopathological and immunohistochemical evaluations were instrumental in tumor classification and characterization.

Granulosa cell tumours were observed in two forms: (1) the predominant form, characterized by intrafollicular radial aggregation of tumour cells around eosinophilic material (Call-Exner bodies) (Fig. 1) and (2) the multifocal coalesced pattern, where lobules of granulosa cells were separated by fibrous tissue. Neoplastic cells in both forms exhibited hyperchromatic nuclei with minimal cytoplasm (Fig. 2).

Two types of cystadenomas were identified: cystadenoma of the ovarian stroma and cystadenoma of the rete ovarii. The former featured neoplastic epithelial cells that were polyhedral in shape, well-oriented, and had round to ovoid nuclei with little cytoplasm and few mitotic

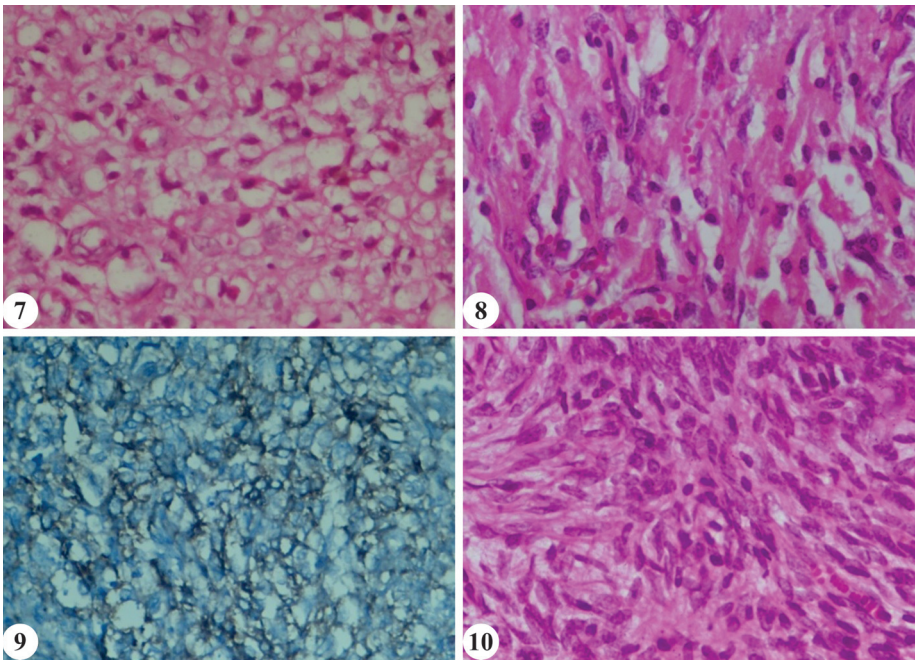

Fig. 7. Clear cell carcinoma: Clear cells with empty cytoplasm and peripherally placed nucleus having a signet ring appearance (H&E x400); **Fig. 8.** Hemangiosarcoma: Proliferated endothelial cells forming clefts supported by thin collagen fibers. The cleft contained few RBCs some without RBCs (H&E x400); **Fig. 9.** Hemangiosarcoma: Cytoplasm of neoplastic endothelial cells showing moderate immunopositivity with VEGF (IHC x400); **Fig. 10.** Fibroma: Spindle shaped fibroblasts with oval nuclei, prominent nuclear borders and dispersed chromatin with multiple nucleoli (H&E x400).

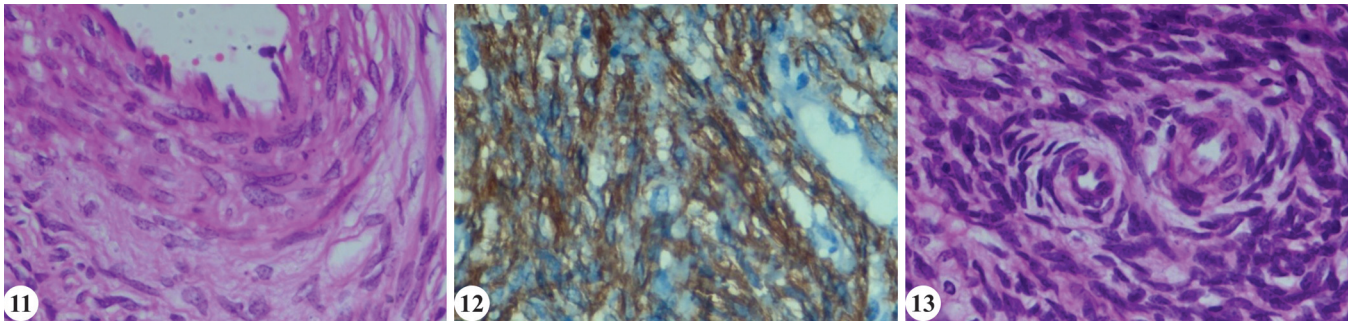

Fig. 11. Angioleiomyoma: Neoplastic smooth muscle cells are proliferating towards the ovarian stroma from the blood vessels (H&E x400); **Fig. 12.** Angioleiomyoma: Immunoexpression of SMA gives a brown colour to the reactive neoplastic cell's cytoplasm and cell outlines (IHC x400); **Fig. 13.** Hemangiopericytoma: Proliferated neoplastic pericytes giving fingerprint like appearance (H&E x400).

figures. Collagen fibers projected into the cystic lumen, with the cystic wall lined by thick collagenous fibers (Fig. 3). The latter, located near the hilus, was characterized by an irregular shape, lined by low cuboidal epithelial cells and enclosed by a fibrous capsule surrounding the cystic space. The neoplastic cells within the cystic space were round to oval in shape, with little cytoplasm and round to elongate nuclei and exhibited few mitotic figures (Fig. 4).

Adenocarcinoma was characterized by neoplastic cells arranged in a tubulo-acinar pattern within a thin supportive connective tissue stroma. The pleomorphic neoplastic cells exhibited round to oval nuclei with scant cytoplasm (Fig. 5). Pan-cytokeratin immunostaining showed mild cytoplasmic positivity in the neoplastic cells, observed as a brown coloration (Fig. 6).

Clear cell carcinoma was identified by cells with clear cytoplasm and peripherally displaced oval to flattened nuclei, giving a typical signet ring appearance (Fig. 7).

Hemangiosarcoma showed as multifocal areas of proliferated and thickened blood vessels with undifferentiated endothelial cells infiltrating the ovarian stroma. The proliferating endothelial cells formed clefts supported by thin collagen fibers. These clefts contained a few red blood cells (RBCs), while some were devoid of RBCs. The proliferating endothelial cells were spherical, ovoid or polygonal in shape, with scant cytoplasm and tapering cytoplasmic processes (Fig. 8). IHC revealed moderate VEGF biomarker immunopositivity in the proliferating endothelial cells (Fig. 9).

Fibroma consisted of whorls and interlacing bundles of mature fibroblasts and collagen within the ovarian stroma. The neoplastic fibrocytes were uniform sized and fusiform in shape, with large nuclei that were normochromatic to hyperchromatic and oval to elongated in shape, containing multiple nucleoli (Fig. 10).

Angioleiomyoma exhibited interlacing bundles of strap-like smooth muscle fibers arranged in an intersecting pattern. The spindle-shaped neoplastic cells had cigar-shaped nuclei with rounded, blunt ends (Fig. 11). Immunostaining for smooth muscle actin (SMA) demonstrated diffuse moderate-to-strong cytoplasmic positivity in myoepithelial cells, smooth muscle cells, myofibroblasts and neoplastic cells (Fig. 12).

Hemangiopericytoma was characterized by neoplastic cells proliferating around blood vessels in a whorl pattern, creating a characteristic fingerprint appearance. The spindle-shaped neoplastic cells had short to moderate cytoplasmic processes. Their nuclei were ovoid to elongated, single and prominent, with some exhibiting chromatin margination along the nuclear membrane (Fig. 13).

DISCUSSION

Ovarian tumours are a common finding in many domestic animals, particularly in bitches and cows[3]. However, due to the practice of culling infertile sheep, the occurrence and characteristics of ovarian neoplasms in this species have not been extensively documented. This study aimed to bridge this knowledge gap by investigating the occurrence and characterizations of different types of ovarian neoplasms in sheep. The findings revealed a significant occurrence of ovarian neoplasms in sheep, comprising one sex cord-stromal neoplasm, three epithelial neoplasms and four mesenchymal neoplasms at the microscopic level. However, none of the ovaries exhibited distinct gross neoplastic changes, which may be attributed to the early culling of the sheep, preventing sufficient time for the development of gross lesions.

The most common neoplastic condition observed was the granulosa cell tumour (GCT), which presented with two distinct histomorphological patterns. The first was the classic form, characterized by radial aggregates of tumor cells surrounding a central deposit of eosinophilic proteinaceous material, known as Call-Exner bodies. This is considered the typical manifestation of GCT[4,5]. The second pattern was multifocal and coalescing, likely representing a combined form of different histological patterns of GCT[9]. In cases of cystadenoma, including cystadenoma of the ovarian stroma and cystadenoma

of the rete ovarii, the neoplastic cells were observed as long papillary fronds or short digitating projections, with some exhibiting a pseudoglandular pattern[3]. The histologic appearance of adenocarcinoma, characterized by proliferating neoplastic cells arranged in a tubulo-acinar pattern with a thin supportive connective tissue stroma, is a consistent finding[10]. The microscopic characterization of the Clear cell carcinoma as polyhedral cells with clear to eosinophilic cytoplasm, peripherally displaced oval to flattened nuclei and diffuse sheets of cells separated by collagenous septa is aligned with the findings of Tavassoli and Devilee[11]. The histological features of Hemangiosarcoma described by Jangir *et al.* are consistent with those found in the current study, specifically being characterized by the presence of clefts formed by undifferentiated endothelial cells. These clefts were supported by thin collagen fibers and sometimes contained red blood cell[12]. According to Moulton, ovarian fibroma is characterized by whorls and interlacing bundles of mature fibroblasts and collagen within the ovarian stroma, a feature corroborated by other authors[5,13]. The same microscopic features of ovarian fibroma were also evident in this study. The microscopic pattern of angioleiomyoma in this study exhibited interlacing bundles of strap-like smooth muscle fibers arranged in an intersecting pattern, as reported previously[14]. The histologic characterization of angioleiomyoma as interlacing bundles of strap-like smooth muscle fibers arranged in an intersecting pattern was reported earlier with similar features[14]. Hemangiopericytoma, observed in this study, appeared as neoplastic cells proliferating around blood vessels in a whorl pattern, creating a fingerprint-like appearance, similar features to those reported in other studies[4,15].

To further characterize these tumours, we performed IHC using specific biomarkers. Hemangiosarcoma showed moderate immunopositivity for VEGF within the proliferating endothelial cells, a finding consistent with previous reports in canine splenic hemangiosarcoma[16]. Sections histologically identified as adenocarcinoma exhibited mild cytoplasmic positivity for pan-cytokeratin within the neoplastic cells, which aligns with the earlier findings on epithelial tumors[17]. Smooth muscle actin demonstrated diffuse moderate-to-strong positivity in myoepithelial cells, smooth muscle cells, myofibroblasts and neoplastic cells in angioleiomyoma, which is consistent with previous studies[18].

This study highlights the significant occurrence and diverse histopathological patterns of ovarian neoplasms in sheep. Our findings, supported by specific immunohistochemical markers such as VEGF, pan-cytokeratin and SMA, provide valuable insights into the characteristics and differentiation of sex cord-stromal, epithelial and mesenchymal ovarian tumours in this species. The results are largely consistent with established literature, emphasizing the importance of detailed pathological examination in diagnosing these conditions in sheep and contributing to the limited body of knowledge on this subject.

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RESEARCH ARTICLE

Hemato-biochemical alterations in Sheep affected with Brucellosis in Anantapur district, Andhra Pradesh, India

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ABSTRACT

Brucellosis, a zoonotic disease caused by *Brucella* spp., significantly impacts the health and productivity of sheep, leading to economic losses and public health concerns. This study evaluated the hematobiochemical alterations in sheep affected with brucellosis in Anantapur district, Andhra Pradesh, India. A total of 100 serum samples from 100 animals (50 male and 50 female) were collected and screened for brucellosis using the Rose Bengal Plate Test (RBPT) and Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA), revealed seroprevalence rates of 16% and 30%, respectively. Hematological and biochemical analyses were conducted on 20 seropositive sheep, with results compared to seronegative controls. Significant hematological changes ($P<0.01$) included reduced hemoglobin, packed cell volume and total erythrocyte count, accompanied by increased total leukocyte count, neutrophils and eosinophils. Lymphocytes, monocytes and basophils were reduced. Biochemical profiles showed elevated aspartate aminotransferase and alanine aminotransferase with decreased total protein and albumin ($P<0.05$).

Keywords: ALT, AST, Brucella, hemoglobin, I-ELISA, neutrophils, PCV, RBPT

INTRODUCTION

Sheep and goats played a crucial role in the livestock sector, serving as vital sources of nutrition and supporting the rural economies of developing nations through their ability to convert forages and residues into valuable products like meat, wool and milk[1]. In India, the sheep population was estimated at 65.07 million and goats at 135.2 million, constituting 12.71% and 26.4% of the total livestock population, respectively[2]. However, small ruminants faced numerous diseases, including brucellosis, a major zoonotic bacterial infection caused by *Brucella spp*., which resulted in substantial economic losses and posed risks to human health[3,4]. Brucellosis transmission occurred through direct contact with infected animals, consumption of contaminated products or exposure to aborted materials, affecting both animals (e.g., abortions, infertility) and humans, particularly farmers and Veterinarians[5,6] . Although bacterial isolation remained the diagnostic gold standard, its limitations-such as the need for BSL-3 facilities-necessitated alternative methods like serological tests (RBPT, ELISA) and PCR, which offered sensitivity and practicality[7-9].

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MATERIALS AND METHODS

The present study was conducted from April to December 2024 in various villages of Anantapur District, Andhra Pradesh, India with sample analyses performed at the Department of Veterinary Pathology, Sri Venkateswara Veterinary University, Tirupati and ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bangalore with prior approval of IAEC under proposal no. 281/go/ReBi/S/2000/CPCSEA/CVSc/TPTY/049/ Veterinary Pathology.

A total of 100 blood samples (male & female each 50) were collected aseptically from the jugular vein of sheep across eight villages. Samples were drawn into serum clot activator tubes, allowed to clot for 2-3 hours and centrifuged at 3000 rpm for 2-4 minutes. Serum was stored at -20^{O}C until analysis.

From seropositive samples, 20 blood samples were selected for hematological analysis using EDTA-collected blood and serum was separated from blood for biochemical analysis using plane vial. Parameters included Hb (Sahli's method), PCV (microhematocrit), TEC and TLC (dilution technique) and DLC (Field's stain, Battlement technique)[10,11]. Biochemical analysis assessed ALT, AST, total protein and albumin in serum centrifuged at 4000 g for 20 minutes, using an Auto Span analyzer with ERBA and Biosystem kits. Statistical

Table 1. Hematological values of sheep affected with brucellosis.

Parameter	Control Sheep (N = 6)	Sheep affected with Brucellosis (N = 20)
Hb - Haemoglobin (g/dl)	10.60 ± 0.20*	7.90 ± 0.20*
PCV - Packed Cell Volume (%)	36.50 ± 0.70*	30.40 ± 0.50*
TEC - Total erythrocyte count (10^6/µl)	10.90 ± 0.45*	4.15 ± 0.18*
TLC - Total leukocyte count (10^3/µl)	5.97 ± 0.45*	22.53 ± 2.52*
MCV - Mean Corpuscular Volume	30.0 ± 0.49*	37.20 ± 0.09*
MCHC - Mean Corpuscular Haemoglobin Concentration	32.40 ± 0.40*	52.70 ± 1.70*
MCH - Mean Corpuscular Haemoglobin	10.60 ± 0.30*	19.60 ± 0.60*
DLC - Differential leukocyte count		
Neutrophils (%)	39.50 ± 0.22*	62.15 ± 2.82*
Lymphocytes (%)	58.17 ± 0.17*	39.33 ± 0.36*
Eosinophils (%)	1.25 ± 0.18*	2.45 ± 0.18*
Monocytes (%)	1.33 ± 0.21*	1.90 ± 0.20*
Basophils (%)	0.58 ± 0.26*	0.83 ± 0.11*
Band cells (%)	0.00 ± 0.00	0.00 ± 0.00

Test: T - Test Significance: (P < 0.05); *Values were significantly different at (P < 0.05)

significance was determined by T-tests for hematological/ biochemical data.

RESULTS

Hematological analysis (Table 1, Fig. 1) showed significant reductions (P < 0.05) in hemoglobin (Hb), packed cell volume (PCV) and total erythrocytes count (TEC) in infected sheep compared to controls, indicating anemia and decreased red blood cell counts. Total leukocytes count (TLC) was significantly elevated (P<0.05) in infected sheep, with differential counts showing significant increases (P<0.05) in neutrophils, eosinophils and monocytes compared to control sheep, while lymphocytes decreased significantly (P<0.05) in positive sheep compared to *Brucella* negative sheep. Basophils elevated slightly in seropositive sheep compared to seronegative sheep (P<0.05). Red blood cell indices also shifted: MCV, MCHC and MCH increased in sheep confirmed with brucellosis compared to control sheep (P<0.05).

Biochemical analysis identified significant changes (P<0.05) in infected sheep with elevated alanine aminotransferase (ALT) in seropositive sheep and increased aspartate aminotransferase (AST) in sheep infected with brucellosis compared to controls, indicating hepatic stress. Significant decrease in total protein and serum albumin was observed in seropositive sheep compared to seronegative sheep (P<0.05), suggesting metabolic disruption.

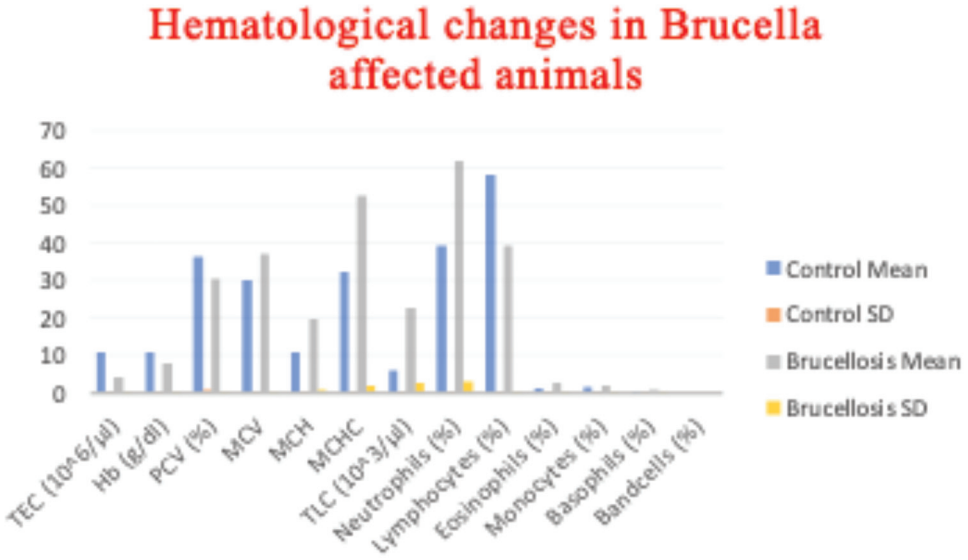

Fig. 1. Hematological values of sheep affected with brucellosis.

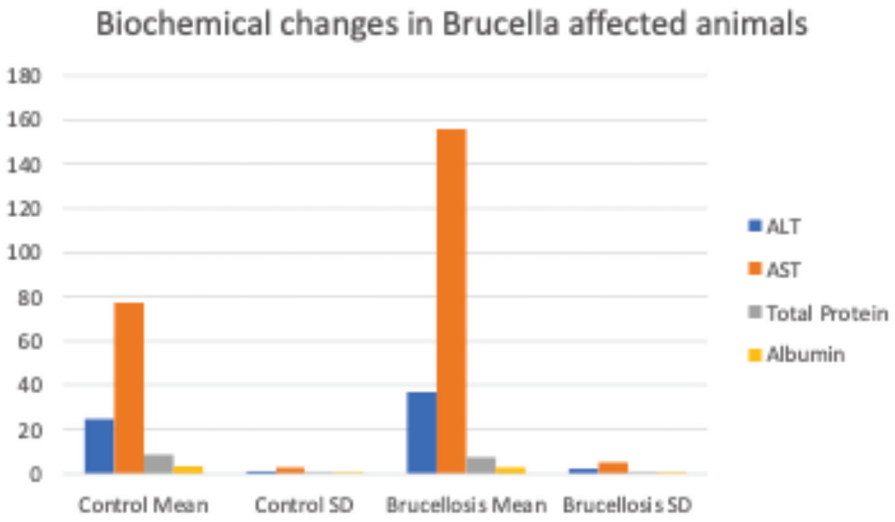

Fig. 2. Serum Biochemical results of Brucella seropositive animals.

DISCUSSION

The hematobiochemical alterations observed in this study revealed the systemic impact of brucellosis on sheep physiology, revealing patterns of anemia, inflammation and hepatic dysfunction. The significant reduction in Hb, PCV and TEC in infected sheep resembled findings by previous reports[12], who documented similar declines in *Brucella*-infected cattle, attributing them to anemia from impaired erythropoiesis or oxidative stress induced by inflammatory cytokines[13]. Previous findings[14,15] also reported anemia in 43.5% and 74% of human brucellosis cases, respectively, supported that Brucella disrupted red blood cell production across species. However, [16]observed stable Hb and PCV in infected cattle, suggesting variability possibly linked to infection stage or host-specific responses.

Table 2. Serum Biochemical results of Brucella seropositive animals.

S.No.	Parameters	Control Sheep (N = 6)	Sheep affected with brucellosis (N = 20)
1.	ALT	24.80 ± 0.77*	36.87 ± 1.86*
2.	AST	77.13 ± 2.70*	155.63 ± 5.16*
3.	Total Protein	8.62 ± 0.18*	7.36 ± 0.11*
4.	Albumin	3.04 ± 0.02*	2.65 ± 0.18*

Test: T - Test Significance: ($P < 0.05$); *Significant level of significance ($P < 0.05$)

The elevated MCV, MCHC and MCH in infected sheep indicated larger, hemoglobin-rich erythrocytes, likely a compensatory mechanism for reduced TEC, as suggested by[17] in cows. This contrasted with[16] who noted stable MCHC in cattle, highlighting potential species differences or dehydration effects in sheep[15]. The marked increase in TLC and neutrophilia reflected an acute inflammatory response aligning with[18], who reported neutrophilia in cattle (26.6%), though at lower levels. The lymphocytopenia in this study agreed with[10] in cattle and human studies[14,15], suggesting immune suppression or bone marrow exhaustion, unlike the lymphocytosis observed in cattle[18] and horses[19]. Elevated eosinophils and monocytes further supported an active immune response, differing from[16], who noted only mild changes in cattle. The slight rise in basophils was less pronounced but consistent with systemic inflammation. These discrepancies likely stemmed from species-specific immune dynamics or the intracellular nature of Brucella, which might suppress certain leukocyte populations while stimulating others[20,16].

Biochemically, the increase in AST and ALT in infected sheep indicated hepatic injury, supporting[21,13,22] who correlated liver enzymes to inflammation and hepatocyte necrosis driven by *Brucella*-induced oxidative stress. The reduced total protein and albumin aligned with[23,21], reflecting impaired hepatic synthesis or renal loss as hypothesized by[24]. In contrary, [25]reported increased total protein in cattle, highlighted species-specific metabolic responses.

CONCLUSION

This study described the hematobiochemical alterations in sheep affected by brucellosis in Anantapur District, revealing a 30% seroprevalence and significant physiological impacts. The marked reductions in Hb, PCV and TEC, coupled with elevated TLC and shifts in leukocyte populations, confirmed anemia and inflammation as hallmarks of the disease in sheep. Elevated AST and ALT was associated with decreased total protein and albumin, highlighting hepatic dysfunction and metabolic disruption, consistent with Brucella's

systemic pathogenicity. These findings aligned with prior research in ruminants and humans, though variations demonstrated species-specific responses and infection stages. The higher sensitivity of I-ELISA over RBPT reinforced its value in epidemiological surveillance. Collectively, these results emphasized brucellosis as a multifaceted disease necessitating integrated diagnostic and management strategies to mitigate its economic and zoonotic toll in India's livestock sector.

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Conflicts of Interest: None

Use of Artificial Intelligence (AI)-Assisted Technology for manuscript preparation: The authors confirm that there was no use of AI-assisted technology for assisting in the writing of the manuscript and no images were manipulated using AI.

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RESEARCH ARTICLE

Studies on Pathological Alterations and Diseases of Nervous System in Small Ruminants

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ABSTRACT

Neurological disorders in small ruminants present a significant challenge to livestock health and productivity, yet they remain comparatively underexplored. This study aimed to characterize the pathological alterations in the central nervous system (CNS) of goats and sheep exhibiting neurological signs. A total of 68 brain samples (44 goats; 24 sheep) were collected during post-mortem examinations. Gross lesions included meningeal congestion, cerebral and cerebellar hemorrhages, encephalomalacia and the presence of Oestrus ovis larvae. Histopathological evaluation revealed inflammatory lesions (vasculitis, meningitis, neuronophagia), reactive changes (chromatolysis, gliosis, satellitosis), vascular alterations (congestion, edema) and degenerative changes (spongiosis, axonal degeneration). Disease specific lesions consistent with listeriosis and rabies were also observed. Bacteriological culture and PCR confirmed *Listeria monocytogenes* and *Escherichia coli* as causative agents in select cases. The findings underscore the diagnostic value of integrating gross pathology, histopathology, microbiology and molecular techniques for the comprehensive identification and understanding of CNS disorders in small ruminants.

Keywords: CNS lesions, *Escherichia coli*, histopathology, *Listeria monocytogenes*, PCR, small ruminants

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INTRODUCTION

Small ruminants, particularly goats and sheep, are integral to India's livestock sector, supporting the livelihoods of marginal and landless farmers due to their adaptability, low maintenance and high reproductive potential. They contribute significantly to food and nutritional security through milk, meat and wool production. As per the 2019 Livestock Census, India had 148.88 million goats and 74.26 million sheep[1], ranking first globally in goat milk production and among the top in small ruminant meat output.

Despite their importance, goats and sheep are highly susceptible to infectious and non-infectious diseases, including neurological disorders, which often go under reported. Contributing factors such as climate change, poor fodder availability and unregulated animal movement have exacerbated disease prevalence[2]. While diseases of the respiratory, digestive and reproductive systems are well studied, CNS disorders remain relatively underexplored. These include listeriosis, coenurosis, polioencephalomalacia, enterotoxaemia and congenital anomalies[3,4].

Listeriosis, a zoonotic disease caused by *Listeria monocytogenes*, is frequently associated with poor-quality silage and has been reported in northern India, including Punjab[5]. Other notable CNS conditions include coenurosis *(Taenia multiceps)* and enterotoxaemia caused by *Clostridium perfringens* type D. Definitive diagnosis relies on post-mortem examination of the brain, with characteristic lesions such as encephalomalacia, gliosis and perivascular cuffing. Ancillary tests like bacterial culture, PCR and immunohistochemistry enhance diagnostic accuracy[6].

This study aimed to investigate the pathological changes in the brains of goats and sheep showing neurological signs, document gross and microscopic lesions, identify etiological agents using conventional and molecular techniques and support improved diagnosis of CNS disorders in small ruminants.

MATERIALS AND METHODS

Sample collection

A total of 68 brain samples were collected from post-mortem examinations of sheep (n = 24) and goats (n = 44) exhibiting neurological signs or with relevant clinical history. The animals were either brought to the Post-mortem Hall of GADVASU or were part of outbreaks on organized and unorganized farms in Punjab. The entire brain was removed aseptically and gross changes were recorded and photographed (GADVASU/2024/IAEC/75/09).

Histopathological examination

Representative brain tissue samples were fixed in 10% neutral buffered formalin (NBF), processed routinely and embedded in paraffin wax. Sections of 3-4 µm thickness were cut using a semi-automated microtome and mounted on glass slides. Haematoxylin and eosin (H&E) staining was performed for routine histopathological evaluation[7]. Selected samples were also subjected to special stains such as Silver-Golgi, Masson's Trichrome, Masson-Fontana and Triple Shorr stains to demonstrate specific tissue elements.

Bacterial isolation and identification

For bacteriological analysis, brain tissue was aseptically inoculated onto Brain Heart Infusion (BHI) agar and incubated at 37^{O}C for 24-48 hours. Pure colonies were sub cultured and identified based on colony morphology and Gram staining[8]. Selective media were used for the isolation of specific pathogens: *Listeria monocytogenes* was cultured on Listeria Selective Agar (HiCrome) and *Escherichia coli* was isolated on Eosin Methylene Blue (EMB) agar.

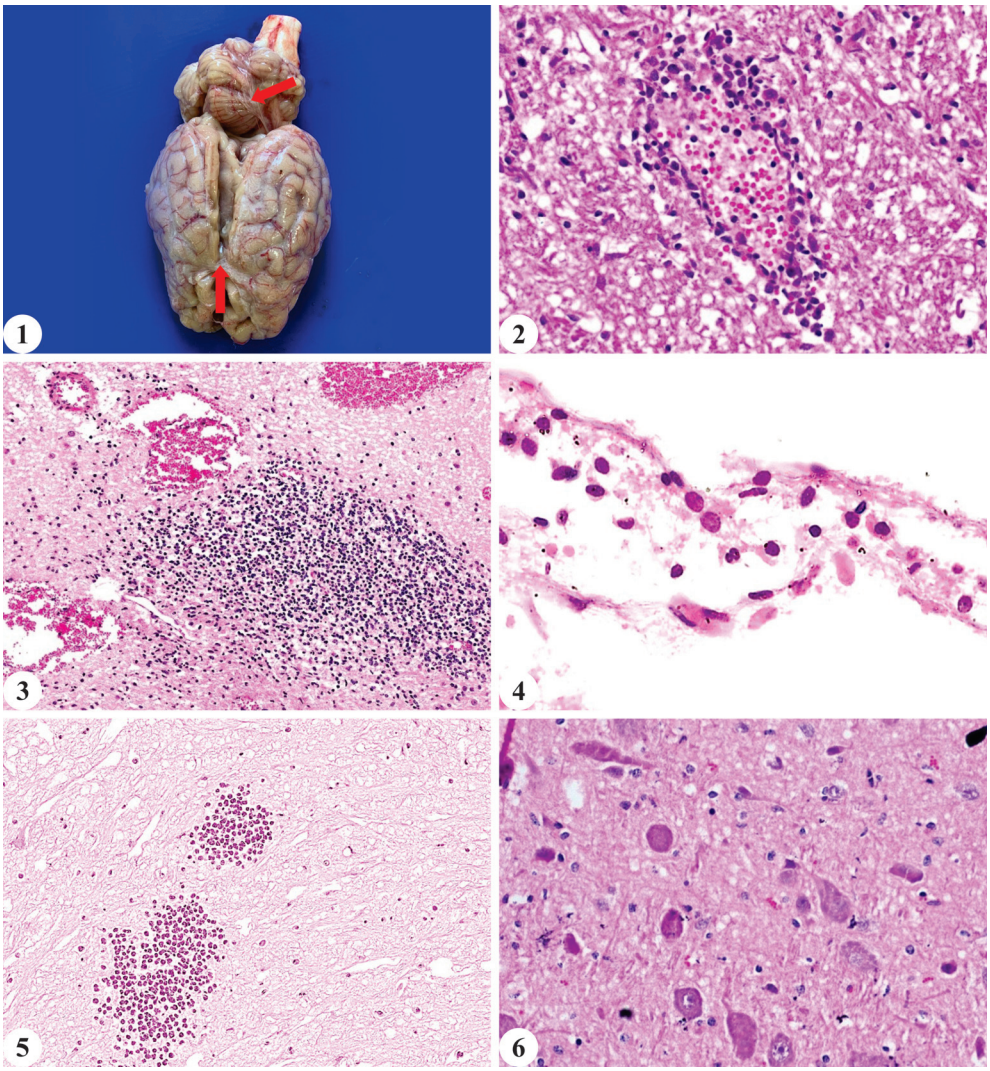

Fig. 1. Fibrinous meningitis with adhesions on the brain surface of goat (arrow); **Fig. 2.** Perivascular cuffing characterized by accumulation of mononuclear inflammatory cells around blood vessels of goat (H&E x400); **Fig. 3.** Non suppurative encephalitis: Cerebellar haemorrhage with lymphocytic infiltration characterized by extravasated red blood cells (haemorrhage) and a dense accumulation of lymphocytes in sheep brain (H&E x200); **Fig. 4.** Meningitis infiltration of mixed cell in goat (H&E x1000); **Fig. 5.** Suppurative encephalitis showing microabscess in brain of sheep (H&E x200); **Fig. 6.** Chromatolysis characterized by a swollen cell body and dispersion of Nissl substance in goat (H&E x400).

Bacteria	Forward Primer	Reverse Primer	Gene	Product Size (bp)
E. coli	CTTACCGGGCAATACACTCACTA	CTTACCGGGCAATACACTCACTA	*phoA*	622
Listeria Monocytogens	ATGAATATGAAAAAAGCAACGATC	TAGCACTTGCACTTGAATTGCTG	*iap*	1120

Molecular diagnosis

DNA was extracted from bacterial isolates using the crude-boiling TENT buffer method[9,10,11] and the DNeasy Blood & Tissue Kit (Qiagen, Germany). Species-specific primers targeting the *phoA* gene for *E. coli* and the *iap* gene for *L. monocytogenes* were used[12]. PCR amplification was performed in a final volume of 25 µl and products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

RESULTS

A total of 68 brain samples, including 44 from goats and 24 from sheep were examined to investigate central nervous system (CNS) disorders through gross pathological, histopathological, bacteriological and molecular analyses.

Gross pathological findings

Gross lesions were observed in 18 of 44 goat brains (40.90%) and 13 of 24 sheep brains (54.17%). Key findings included meningeal congestion (6 goats, 33.3%; 3 sheep, 23.07%), fibrinous adhesions (Fig. 1) (4 goats, 22.2%; 2 sheep, 15.38%), cerebral hemorrhages (2 goats, 11.1%; 3 sheep, 16.6%) and cerebellar hemorrhages (4 goats, 30.76%; 2 sheep, 15.38%). Encephalomalacia was recorded in 2 goats (11.1%) and 1 sheep (7.69%). Oestrus ovis larvae were found in the cranial cavities of one goat (5.55%) and one sheep (7.69%) highlighting incidental parasitic infestation.

Histopathological findings

Neuropathological lesions were detected in 35 goat brains and 17 sheep brains. Lesions were classified as inflammatory, reactive, vascular, degenerative and disease specific.

Inflammatory lesions

Inflammatory changes were present in 20 goats (45.45%) and 9 sheep (37.5%). Vasculitis was observed in 4 goat brains (20%) and 2 sheep brains (22.2%). Perivascular cuffing (Fig. 2) occurred in 6 goats (30%) and 3 sheep (33.3%). Neuronophagia (6 goats, 30%; 2 sheep, 22.2%) and microgliosis (4 goats, 20%; 3 sheep, 33.3%) were also noted. Non-suppurative encephalitis (Fig. 3) was seen in 3 goats (15%) and 2 sheep (22.2%), while suppurative encephalitis was detected in 2 cases each of both species. Meningitis was noted in 3 goats and 2 sheep (Fig. 4) and ependymitis in 2 goats and 1 sheep.

Reactive changes

Reactive alterations were observed in 15 goats (34.09%) and 7 sheep (29.17%). Chromatolysis was the most frequent reactive change in goats (6 cases, 40%) but was infrequent in sheep (1 case, 14.2%). Satellitosis was more prominent in sheep (3 cases, 42.8%) than in goats (5 cases, 33.3%) (Fig. 7). Gliosis was equally frequent in both species (4 goats, 26.6%; 3 sheep, 42.8%).

Vascular lesions

Vascular lesions were recorded in 20 goats (45.45%)

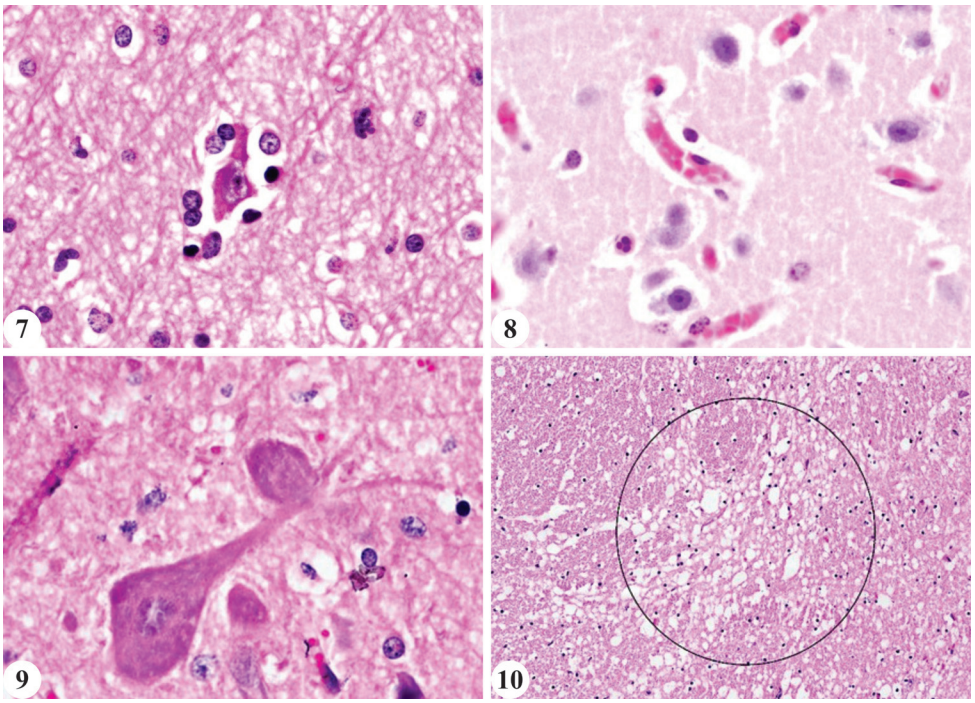

Fig. 7. Satellitosis: Several smaller cells with darkly stained, condensed nuclei are clustering around the neuron in sheep (H&E x1000); **Fig. 8.** Perivascular edema characterized by clear halos surrounding neuronal cell bodies and blood vessels of goat (H&E x1000); **Fig. 9.** Axonal spheroid: Rounded, eosinophilic swellings within the neuropil of sheep (H&E x1000); **Fig. 10.** Spongiosis: Multiple clear vacuoles or empty spaces in the neuropil of goat (H&E x200).

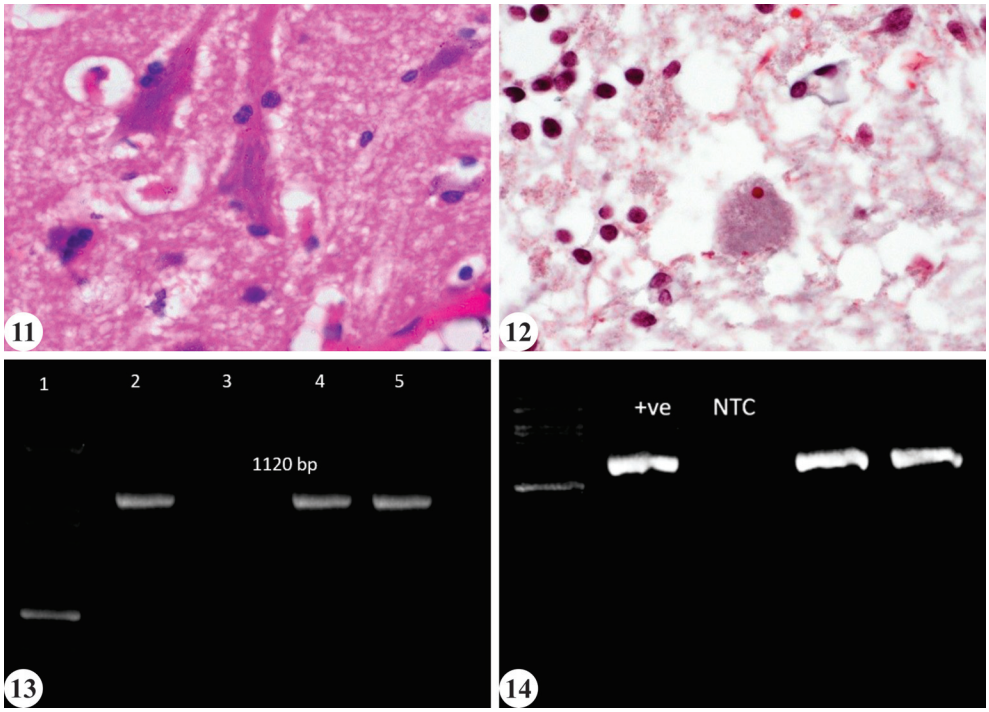

Fig. 11. Neurofibrillary tangle dense, brightly eosinophilic (pink-red) fibrillary mass within the cytoplasm of the neuron in sheep (H&E x1000); **Fig. 12.** Rabies: Large intracytoplasmic Negri body within a neuron of goat (Triple Shorr stain x1000); **Fig. 13.** Agarose gel electrophoresis. PCR analysis for sample suspected for *L. monocytogenes*, Lane 1: 100 bp DNA ladder, Lane 2: Positive control (1120 bp), Lane 3: Negative control, Lane 4 & 5: Samples; **Fig. 14.** Agarose gel electrophoresis. PCR analysis for sample suspected for *E. coli*, Lane 1: 100 bp DNA ladder, Lane 2: Positive control (622 bp), Lane 3: Negative control, Lane 4 & 5: Samples.

and 9 sheep (37.5%). Haemorrhages were found in 14 goat brains (70%) and 8 sheep brains (88.8%), while congestion was present in 13 goats (65%) and all 9 sheep (100%). Vasogenic oedema was identified in 8 goats (40%) and 7 sheep (77.7%). Perineuronal, perivascular and perixonal oedema was noted in 5 goats (25%) and 4 sheep (44.4%) (Fig. 8).

Degenerative lesions

Degenerative changes were seen in 9 goat brains (20.45%) and 6 sheep brains (25%). Axonal spheroids were seen in 1 case each (11.1% goats; 16.6% sheep) (Fig. 9). Spongiosis was observed in 3 goats and 2 sheep (33.3% each) (Fig. 10). Neuronal degeneration (2 goats, 22.2%; 1 sheep, 16.6%) and vacuolation (2 goats, 22.2%; 1 sheep, 16.6%) were also recorded (Fig. 3). Neurofibrillary tangles were found in one goat and one sheep (Fig. 11).

Disease specific lesions

Listeriosis was diagnosed in one goat and one sheep, exhibiting non-suppurative meningoencephalitis with microgliosis and perivascular cuffing, especially in the brainstem. Rabies was confirmed in one goat and one sheep, characterized by neuronal degeneration, perivascular lymphocytic cuffing, babe's nodule and Negri bodies (Fig. 12).

Other lesions

Rare findings included meningeal melanosis in one goat and one sheep and neoplastic growths in another goat and sheep. These cases suggest potential congenital pigmentary anomalies or rare intracranial neoplasms.

Bacteriological and molecular findings

Bacterial culture identified *Listeria monocytogenes* and *Escherichia coli* in 2% of goat brains and 5.55% of sheep brains, respectively. PCR confirmed their identity using the *iap* gene for *L. monocytogenes* (Fig. 13) and *phoA* gene for *E. coli* (Fig. 14).

DISCUSSION

This study highlights the diverse spectrum of CNS pathologies in small ruminants. Gross lesions, including meningeal congestion, hemorrhages and encephalomalacia were more frequently observed in sheep, suggesting a potentially higher susceptibility or advanced disease progression at the time of death. Histopathologically, inflammatory changes predominated, with perivascular cuffing, microgliosis and neuronophagia indicating active encephalitic processes. These lesions, especially prominent in the brainstem and cortex are consistent with bacterial infections such as listeriosis or coliform septicemia aligning with previous reports[13]. Reactive gliosis, chromatolysis and satellitosis were common and reflect the CNS's attempt to counteract injury. Chromatolysis in goats

may indicate sustained neuronal stress, possibly from chronic infection or metabolic dysfunction[14]. Vascular disturbances such as congestion and haemorrhages, observed in nearly all sheep and many goats, suggest significant cerebrovascular compromise. Vasogenic edema and associated perivascular/perineuronal swelling underscore disruptions to the blood-brain barrier frequently seen in septic or toxic encephalopathies.

Degenerative lesions, while less frequent were noteworthy. Spongiosis and neuronal vacuolation suggest metabolic or toxic causes like polioencephalomalacia. The detection of neurofibrillary tangles in both species, though rare is notable and suggests possible age-related brain degeneration in ruminants[15]. Disease specific diagnoses were confirmed in a few cases through molecular techniques, including PCR. *Listeria monocytogenes* was detected in samples exhibiting classical signs of non-suppurative meningoencephalitis, including micro abscess formation and extensive perivascular cuffing. These findings were in line with the neuropathological hallmarks described in earlier studies. Similarly, rabies was confirmed in two animals one goat and one sheep based on the presence of Negri bodies, gliosis, babe's nodule and neuronophagia. These lesions support existing descriptions in the literature[16,17]. Rare lesions such as meningeal melanosis and CNS neoplasia although incidental, underscore the importance of comprehensive neuropathological assessments. These findings also hint at the broader spectrum of neuropathological entities in small ruminants some of which may go unnoticed in routine clinical diagnosis[18].

In conclusion, the high prevalence and variety of lesions observed in both goats and sheep highlight the diagnostic value of postmortem CNS evaluation. Integration of histopathology with bacteriological and molecular tools is critical for accurate diagnosis, epidemiological tracking and understanding disease pathogenesis in small ruminant neurology.

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RESEARCH ARTICLE

Studies on erythrocytic abnormalities with leukogram and cytological findings of dogs

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ABSTRACT

The study was conducted on blood samples of dogs to correlate the erythrocytic abnormalities with leukogram and cytological findings. The study was conducted on blood samples submitted to Centralized Clinical Diagnostic Laboratory in the Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) Ludhiana. The Leukogram analysis was carried out in all the 363 cases and detailed leukocytic abnormalities were recorded. The cases were categorized into those having normal total leukocyte count, leukopenia, leukocytosis and finally the cases having leukemoid response. On the basis of cytological examination, the cases were classified into neoplastic and non-neoplastic conditions. A good correlation was between presence of codocytes, schistocytes, echinocytes, polychromatophilic RBCs and leukocytosis and stomatocytes and polychromatophilic RBCs with leukopenia. In addition, acanthocytes, echinocytes and schistocytes were common in cases of lymphoma.

Keywords: Blood, correlation, cytology, dogs, erythrocytic abnormalities, leukogram

INTRODUCTION

Hematological examination is an important diagnostic tool and it is often used to correlate the physical examination and the medical history for diagnosis of diseases in both medical and veterinary practice[1]. Routine haematological examination includes complete blood cell count and blood smear examination for leukocytic and erythrocytic abnormalities[2,3]. Even in the age of molecular analysis, the blood smear remains an important diagnostic tool[4]. Erythrocyte morphological abnormalities have been reported to be associated with disease conditions. Morphology abnormalities like acanthocytes, schistocytes spherocytosis have been reported to be associated with different disease conditions in dogs[5,6]. However, anemia with erythropenia, without significant changes in erythrocyte indices, thrombocytopenia and leukocytosis and slight variation in differential white blood cell count was studied in dogs with mammary gland carcinoma[7]. In addition, canine lymphoma was the most common hematologic malignancy in dogs and in many aspects comparable to non-Hodgkin lymphoma in humans characterized by the involvement of multiple lymph nodes and the infiltration of various organs especially liver and spleen by neoplastic lymphocytes[8]. Therefore, the present study was undertaken to correlate the erythrocytic abnormalities with the leukocytic and cytological changes in dogs.

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MATERIAL AND METHODS

The present study was conducted on blood samples of dogs presented to Centralized Clinical Diagnostic Laboratory in the department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) Ludhiana, with effect from 1st July 2018 to 31st March 2019. A total of 363 cases of dogs were analyzed. Out of these, 259 were analyzed manually and 104 cases were analyzed using ADVIA 2120 Hematology System (Siemens, USA). Leukogram analysis was carried out in all 363 cases and detailed leukocytic abnormalities were recorded. The cases were categorized into those having normal total leukocyte count, leukopenia, leukocytosis and finally the cases having leukemoid response. Moreover, cytological examination of the smears submitted to the Centralized Clinical Diagnostic Laboratory was also performed. The smears were stained by Leishman stain. Based on cytological examination, the cases were classified into neoplastic and non-neoplastic conditions.

Statistical analysis

The data pertaining to different parameters was analyzed using Statistical Analysis Software (SAS-version 9.3, Institute, Cary, USA). The comparison between

Table 1. Correlation of morphological abnormalities of erythrocytes with leukogram findings.

Erythrocytic morphology	Normal TLC	Leukopenia	Leukocytosis	Leukemoid response
Acanthocytes	$6.23^{a} \pm 1.35$	$5.11^{a} \pm 2.91$	$9.13^{a} \pm 1.90$	$10.16^{a} \pm 6.86$
Blister cells	$0.76^{a} \pm 0.24$	$0.11^{a} \pm 0.11$	$0.24^{a} \pm 0.12$	$0.00^{a} \pm 0.00$
Codocytes	$30.88^{a} \pm 3.01$	$24.96^{a} \pm 5.60$	$36.68^{a} \pm 3.39$	$48.33^{a} \pm 7.89$
Dacrocytes	$1.23^{a} \pm 0.31$	$2.25^{a} \pm 1.26$	$0.75^{a} \pm 0.25$	$0.00^{a} \pm 0.00$
Echinocytes	$45.09^{a} \pm 5.29$	$38.18^{a} \pm 9.89$	$52.58^{a} \pm 5.73$	$18.5^{a} \pm 12.35$
Elliptocytes	$0.11^{a} \pm 0.11$	$0.00^{a} \pm 0.00$	$0.86^{a} \pm 0.85$	$0.00^{a} \pm 0.00$
Incomplete spherocytes	$11.83^{a} \pm 2.06$	$19.51^{a} \pm 13.39$	$7.29^{a} \pm 1.39$	$0.00^{a} \pm 0.00$
Keratocytes	$0.23^{a} \pm 0.08$	$0.37^{a} \pm 0.17$	$0.24^{a} \pm 0.12$	$0.00^{a} \pm 0.00$
Nucleated RBCs	$0.15^{a} \pm 0.08$	$0.07^{a} \pm 0.07$	$0.20^{a} \pm 0.07$	$0.00^{a} \pm 0.00$
Polychromatophils	$1.65^{a} \pm 0.44$	$0.40^{a} \pm 0.19$	$4.01^{a} \pm 0.72$	$4.5^{a} \pm 4.11$
Quatrefoil RBCs	$0.87^{a} \pm 0.17$	$0.40^{a} \pm 0.31$	$0.90^{a} \pm 0.18$	$1.5^{a} \pm 0.84$
Schistocytes	$18.36^{a} \pm 2.26$	$12.11^{a} \pm 2.55$	$26.83^{a} \pm 3.15$	$28^{a} \pm 7.22$
Spherocytes	$50.84^{a} \pm 3.39$	$41.40^{a} \pm 11.53$	$39.13^{a} \pm 3.63$	$25.16^{a} \pm 6.78$
Stomatocytes	$0.75^{a} \pm 0.21$	$1.81^{a} \pm 0.74$	$1.11^{a} \pm 0.30$	$0.00^{a} \pm 0.00$

Values with different superscript (a, b, c) within a parameter differ significantly ($p<0.05$)

the normally distributed quantitative variables was done using ANOVA along with Duncan correction. In addition, correlation and regression analysis was also carried out and the association between different erythrocytic abnormalities and leukogram findings was calculated.

RESULTS

In the present study, the erythrocytic abnormalities observed were correlated with the findings of the leukogram in all 363 cases (Table 1). The leukogram was classified into animals having normal TLC, leukopenia, leukocytosis and leukemoid response. The mean value of acanthocytes was more in dogs showing leukemoid response, followed by those having leukocytosis, normal TLC and leukopenia respectively. The mean values of blister cells were more in dogs showing normal TLC, followed by leukocytosis and no blister cells were observed in leukopenia respectively. The mean value of codocytes was more in dogs showing leukemoid response, followed by leukocytosis, normal TLC and leukopenia. The mean value of dacrocytes was more in dogs showing leukopenia, followed by normal TLC, leukocytosis. The mean value of echinocytes was more in dogs showing leukocytosis, followed by normal TLC and no echinocytes were observed in leukopenia and leukemoid response. The mean value of elliptocytes was more in dogs showing leukocytosis, followed by normal TLC, no elliptocytes were observed in leukemoid response. The mean value of incomplete spherocytes was more in dogs showing leukopenia, followed by normal TLC and leukocytosis, no incomplete spherocytes were observed in leukemoid response. The mean value of keratocytes was more in dogs showing leukopenia, followed by leukocytosis and normal TLC, no keratocytes were observed in cases showing leukemoid response. The mean value of nucleated RBCs was more in dogs showing leukocytosis, followed by normal TLC and leukopenia, no nucleated RBCs were observed in cases having leukemoid response. The mean value of polychromatophilic RBCs was more in dogs showing leukemoid response, followed by leukocytosis, normal TLC and leukopenia. The mean value of q RBC was more in dogs showing leukemoid response, followed by leukocytosis, normal TLC and leukopenia. The mean value of schistocytes was more in dogs showing leukemoid response, followed by leukocytosis, normal TLC and leukopenia. The mean value of spherocytes was more in dogs showing normal TLC, followed by leukopenia, leukocytosis and leukemoid response. The mean value of stomatocytes was more in dogs showing leukopenia, followed by leukocytosis, normal TLC respectively and no stomatocytes were observed in leukemoid response. The mean values of acanthocytes, blister cells, codocytes, dacrocytes, echinocytes, elliptocytes, incomplete spherocytes, keratocytes, neucleated RBCs, polychromatophilic RBCs, q RBCs, schistocytes, spherocytes and stomatocytes did not differ significantly in cases having normal TLC, leukopenia, leukocytosis and leukemoid response.

Table 2 depicts the correlation between serum chemistry findings with the findings of leukogram. The mean value of total protein was more in dogs showing leukopenia, followed by normal TLC, leukocytosis and leukemoid response. The mean value of albumin was more in dogs showing normal TLC, followed by leukocytosis, leukemoid response and leukopenia. The mean value of ALT was more in dogs showing leukocytosis, followed by normal TLC, leukopenia and leukemoid response. The mean value of AST was more

Table 2. Correlation of serum chemistry findings with the leukogram findings.

Parameter	Units	Normal TLC	Leukopenia	Leukocytosis	Leukemoid response
Total protein	(g/dL)	5.53^{a} ± 0.11	5.48^{a} ± 0.31	5.09^{a} ± 0.11	4.58^{a} ± 0.42
Albumin	(U/L)	2.21^{a} ± 0.05	1.89^{a} ± 0.08	2.07^{a} ± 0.05	1.98^{a} ± 0.45
Alanine aminotransferase (ALT)	(U/L)	93.69^{a} ± 10.07	88.42^{a} ± 33.56	129.62^{a} ± 15.68	66^{a} ± 18.30
Aspartate aminotransferase (AST)	(U/L)	75.85^{a} ± 7.63	102.13^{a} ± 58.03	105.51^{a} ± 12.36	103.5^{a} ± 43.95
Gamma-glutamyl transferase (GGT)	(U/L)	11.30^{a} ± 2.29	21.13^{a} ± 14.71	21.72^{a} ± 4.69	15.75^{a} ± 6.23
Total bilirubin	(mg/dL)	0.40^{b} ± 0.05	0.89^{b} ± 0.58	1.82^{b} ± 0.35	8.15^{a} ± 5.34
Alkaline phosphatase (ALKP)	(U/L)	242.17^{b} ± 24.98	270.29^{b} ± 73.94	348.61^{b} ± 36.25	2800.17^{a} ± 2409.03
Blood urea nitrogen (BUN)	(mg/dL)	66.47^{a} ± 10.62	65.02^{a} ± 10.62	50.60^{a} ± 4.10	54.33^{a} ± 21.31
Creatinine	(mg/dL)	5.95^{a} ± 0.49	4.70^{a} ± 1.11	3.63^{a} ± 0.34	3.68^{a} ± 1.47

Values with different superscripts (a, b, c) within parameter differ significantly ($p<0.05$)

in dogs showing leukocytosis, followed by leukemoid response, leukopenia and normal TLC. The mean value of GGT was more in dogs showing leukocytosis, followed by leukopenia, leukemoid response and normal TLC. The mean value of total bilirubin was more in dogs showing leukemoid response, followed by leukocytosis, leukopenia and normal TLC. The mean value of ALKP was more in dogs showing leukemoid response, followed by leukocytosis, leukopenia and normal TLC. The mean value of BUN was more in dogs showing normal TLC, followed by leukopenia, leukemoid response and leukocytosis. The mean value of creatinine was more in dogs showing normal TLC, followed by leukopenia, leukemoid response and leukocytosis. The mean values of total protein, albumin, ALT, AST, GGT, total bilirubin, ALKP, BUN and creatinine did not differ significantly in cases having normal TLC, leukopenia, leukocytosis and leukemoid response.

Table 3 depicts the correlation of hematological findings using hematological analyzer with findings of the leukogram. The mean value of anisocytosis was more in dogs showing leukocytosis, followed by normal TLC, and no anisocytosis were observed in leukopenia and leukemoid response respectively. The mean value of hypochromasia was more in dogs showing leukemoid response, followed by normal TLC and leukocytosis, and no hypochromasia were observed in leukopenia respectively. The mean value of polychromasia was more in dogs showing leukemoid response, followed by normal TLC and leukocytosis and no polychromasia were observed in leukopenia respectively. The mean value of

Table 3. Correlation of hematological findings using hematological analyzer with the findings of the leukogram (n=104).

Variables	Normal TLC	Leukopenia	Leukocytosis	Leukemoid response
Anisocytosis	0.14^{a} ± 0.07	0.00^{a} ± 0.00	0.16^{a} ± 0.09	0.00^{a} ± 0.00
Hypochromasia	0.06^{a} ± 0.03	0.00^{a} ± 0.00	0.06^{a} ± 0.05	0.25^{a} ± 0.25
Polychromasia	0.12^{a} ± 0.04	0.00^{a} ± 0.00	0.04^{a} ± 0.03	0.25^{a} ± 0.25
Normocytic	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00
Microcytic	0.5^{a} ± 0.12	0.5^{a} ± 0.37	0.30^{a} ± 0.11	0.00^{a} ± 0.00
Macrocytic	0.04^{a} ± 0.02	0.00^{a} ± 0.00	0.09^{a} ± 0.07	0.00^{a} ± 0.00
CH (Cellular Hemoglobin)	20.20^{a} ± 0.37	19.37^{a} ± 0.37	19.94^{a} ± 0.40	19.5^{a} ± 0.62
CHCM (Corpuscular Hb concentration mean)	32.49^{a} ± 0.28	33.0^{a} ± 0.73	32.08^{a} ± 0.46	33.07^{a} ± 1.12
HCT	31.68^{a} ± 1.52	26.35^{a} ± 2.54	29.44^{a} ± 1.50	28.3^{a} ± 4.16
HDW (Hb concentration distribution width)	2.44^{a} ± 0.13	2.14^{a} ± 0.16	2.68^{a} ± 0.16	2.66^{a} ± 0.31
MCH	19.17^{a} ± 0.55	16.38^{a} ± 1.49	18.90^{a} ± 0.58	15.1^{a} ± 2.34
MCHC	31.04^{a} ± 0.89	27.9^{a} ± 2.40	29.98^{a} ± 1.03	25.57^{a} ± 4.04
MCV	62.23^{a} ± 1.04	58.75^{a} ± 1.38	63.22^{a} ± 1.26	45.85^{b} ± 13.38
MPV	13.47^{a} ± 0.87	13.3^{a} ± 2.04	14.07^{a} ± 0.95	14.4^{a} ± 2.54
RBC	5.18^{a} ± 0.26	4.53^{a} ± 0.45	4.70^{a} ± 0.23	4.82^{a} ± 0.80
RDW	15.01^{a} ± 0.38	14.72^{a} ± 0.51	16.35^{a} ± 0.58	17.3^{a} ± 2.12
WBC	12.74^{a} ± 1.45	4.37^{a} ± 0.47	29.07^{a} ± 1.94	72.22^{a} ± 30.95

Values with different superscripts (a, b, c) within a parameter differ significantly ($p<0.05$)

microcytosis was more in dogs showing normal TLC and leukopenia, followed by leukocytosis and no microcytosis were observed in leukemoid response. The mean value of macrocytosis was more in dogs showing leukocytosis followed by normal TLC and no macrocytosis were observed in leukopenia and leukemoid response. The mean value of CH was more in dogs showing normal TLC, followed by leukemoid response, leukocytois and leukopenia. The mean value of CHCM was more in dogs showing leukemoid response, followed by leukopenia, normal TLC and leukocytosis. The mean value of HCT was more in dogs showing normal TLC, followed by leukocytosis, leukemoid response and leukopenia. The mean value of HDW was more in dogs showing leukocytosis followed by leukemoid response, normal TLC and leukopenia. The mean value of MCH was more in dogs showing normal TLC followed by leukocytosis, leukopenia and leukemoid response. The mean value of MCHC was more in dogs showing normal TLC followed by leukocytosis, leukopenia and leukemoid response. The mean value of MCV was more in dogs showing leukocytosis followed by normal TLC, leukopenia and leukemoid response. The mean value of MPV was more in dogs showing leukocytosis followed by leukemoid response, normal TLC and leukopenia. The mean value of RBC was more in dogs showing normal TLC followed by leukemoid response, leukocytosis and leukopenia. The mean value of RDW was more in dogs showing leukemoid response followed by leukocytosis, normal TLC and leukopenia. The mean values of anisocytosis, hypochromasia, polychromasia, normocytic RBC, microcytic RBC, macrocytic RBC, CH, CHCM, HCT, HDW, MCH, MCHC, MCV, MPV, RBC, RDW and WBC did not differ significantly in cases having normal TLC, leukopenia, leukocytosis and leukemoid response.

In the present study, correlation between erythrocytic abnormality with leukogram finding was carried out ($p<0.05$) and there was significant correlation between normal TLC with the presence of spherocytes and polychromatophilic RBCs in the blood smears, between leukocytosis and the presence of codocytes, schistocytes, echinocytes and polychromatophilic RBCs in the blood smears. In addition, leukopenia correlated with the presence of stomatocytes and polychromatophilic RBCs in blood smears of dogs.

Correlation between spherocytes and q RBCs were found in TVT cases (Fig. 1 & 2) and echinocytes and schistocytes were found in lymphoma cases, acanthocytes were found in malignant melanoma whereas, acanthocytes and schistocytes were observed in malignant histiocytoma. The codocytes was observed in chronic active inflammation (Fig. 3 & 4).

In the present study, correlation between history and clinical signs with leukogram finding was also carried out ($p<0.05$) and there was significant correlation between

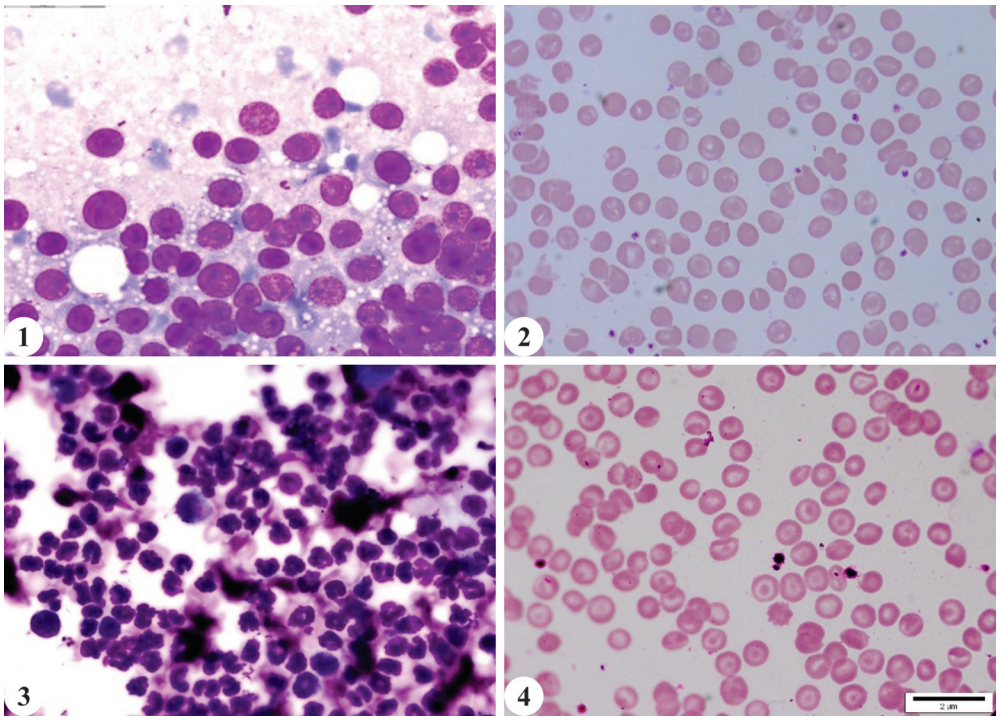

Fig. 1. Impression smear from tumor growth showing round, slightly pleomorphic cells having vacuolated cytoplasm, suggestive of TVT (Leishman stain x100); **Fig. 2.** Blood smear shows Quatrefoil RBCs in dog with TVT (Leishman stain x100); **Fig. 3.** FNAB showing large number of neutrophils and few macrophages, suggestive of chronic active inflammation (Leishman stain x100); **Fig. 4.** Blood smear shows codocytes in dog with chronic active inflammation (Leishman stain x100).

leukocytosis with the presence of clinical signs like blood in vomit, leukopenia with the history of presence of ticks, icteric mucus membrane and distented abdomen.

DISCUSSION

In accordance with our present study, earlier workers have also reported that codocytes and leukemoid response was observed in dogs having renal carcinoma[9]. Scientist observed that echinocytes and leukocytosis was reported in dogs having hemangiosarcoma[10]. Similarly, some researchers reported that hypoalbuminemia, elevated ALT, ALKP levels and leukocytosis was observed in dogs having IMHA[11]. Moreover, it is reported that hypochromasia, leukemoid response and codocytes were associated with chronic granulocytic leukemia in dogs[12], low RBC count, leucopenia, thrombocytopenia was associated with Ehrlichia and Anaplasma infection in dogs[13]. Researcher reported that high WBC counts and leukemoid response was associated with dogs having pyometra[14]. Anisocytosis, polychromasia, codocytes and leukocytosis were observed in dogs having renal carcinoma[9]. Reports of[15] revealed dog having renal failure showed clinical sign of blood in vomit along with leukocytosis. Leukopenia was observed in early cases of canine babesiosis having history of inappetence and ticks infestation[14]. Scientists reported that acanthocytes and schstocytes were observed in blood smear of dogs having lymphoma[16]. Earlier workers reported that dog with malignant melanoma showed acanthocytes and codocytes in blood smears[17]. Codocytes and acanthocytes were seen in blood smear of dogs suffering from chronic kidney diseases[18].

CONCLUSION

From the study it was concluded that erythrocytic abnormalities may provide some insight regarding changes in the leukogram and cytological diagnosis of dogs.

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RESEARCH ARTICLE

Hepatoprotective impact of combined *Emblica urinaria* (L.), *Saussurea costus* and *Rheum webbianum* extracts against diethylnitrosamine (DEN) induced hepatic injury in Wistar rats

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ABSTRACT

Liver diseases have become one of the major causes of morbidity and mortality in men and animals all over the globe and hepatotoxicity due to drugs appears to be the most common contributing factor. A single drug cannot be effective against all types of severe liver diseases. The different plants exhibit different modes of action and in combination, that activity can be enhanced as compared to the single plant extract. The present study was planned to observe the *in vitro* cytotoxicity of 70% aqua ethanolic extracts of the plants namely *Emblica urinaria* (L.) (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) on human oral cancer KB cells, human lung cancer A549 cells and human cervical carcinoma SiHA cells, respectively followed by *in vivo* experimental study in Albino Wistar rats. In the *in-vivo* experimental protocol, a total of 42 rats were randomly divided into 6 groups, where, group I served as plain control and group II was provided with N-diethylnitrosamine (DEN) alone. Group III received DEN with silymarin and groups IV, V and VI were administered with a combination of *Emblica urinaria* (L.), *Saussureacostus* and *Rheum webbianum* extracts in 3 doses in the ratio of 1:2:2 to study growth response, mortality pattern, clinical signs, biochemical and pathological changes against DEN-induced hepatic damage in rats. *Emblica urinaria* (L.), *Saussureacostus* and *Rheum webbianum* extracts have shown maximal % cytotoxicity at their highest concentration i.e. 200 µg/ml. The minimal alterations in the values of liver enzymes (ALT, AST), total protein and creatinine levels, gross and histopathological changes in the ameliorative groups depicted the protective efficacy of these plant extracts in combination as compared with the group II treated with DEN alone. The present study warrants the hepatoprotective potential of the plant mentioned above combinations and signifies the need to use these medicinal plants as a therapeutic regimen in future.

Keywords: Hepatoprotection, liver, medicinal plants, pathology

INTRODUCTION

The liver is one of the most important vital organs concerned with the metabolism of toxins and drugs[1]. Hepatocytes play an important role in our body such as protein synthesis and storage, carbohydrate and lipid metabolism, glycogen storage, cholesterol, bile salts and hormone production, metabolism and detoxification of exogenous (drugs/insecticides) and endogenous (steroids) substances[2]. Several pharmacological agents, while essential for the therapeutic management of various ailments are frequently implicated in hepatocellular damge owing to their biotransformation primarily in the liver and to lesser extent, the kidneys with hepatotoxicity representing a prevalent dose and duration-dependent adrvese effect[3]. In addition, many pollutants are known to be toxic to the liver, such as organic toxicants and heavy metals. Furthermore, liver injuries can develop into fatty liver, hepatitis, fibrosis, cirrhosis, liver failure or even cancers. Hepatocellular carcinoma is one of the most common types of malignant tumours, which represents the third leading cause of death due to cancer and the fifth most prevalent malignancy worldwide[4].

N-diethylnitrosamine (DEN), is a nitrosamine compound used in the experimental research in the laboratory animals to induce hepatic damage and hepatocellular carcinoma. It is found in very trace amounts as a contaminant in some industrial processes, processed meats, smoke or alcoholic beverages, especially those involving nitrites/nitrates and high-temperature processing. Therefore, the search for compounds or drugs to be used as a complementary therapy alongside DEN administration helps reduce DEN-induced

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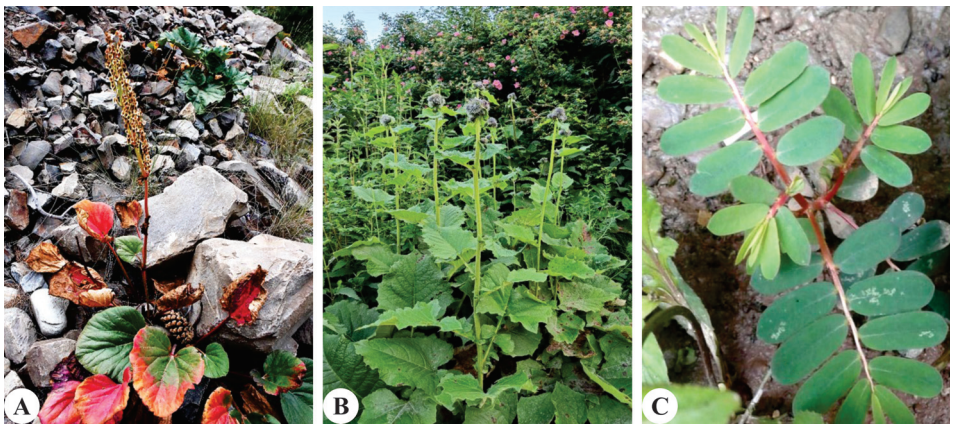

Fig. 1A. *Rheum webbianum*. **B.** *Saussurea costus*. **C.** *Emblica urinaria* (L.).

hepato-toxicity[5]. DEN is a perilous chemical which develops liver cirrhosis and is generally used to initiate hepatocarcinogenesis in rats[6]. DEN is one of the most important hepatocarcinogens and DEN-induced hepatocarcinogenesisis similar to those of human hepatocellular carcinoma (HCC)[7].

Emblica urinaria (L.), one of the herbal plants belonging to the genus *Emblica* (Euphorbiaceae), is widely distributed in China, Southern India and Southern America. *P. Urinaria* is used for the treatment of diseases such as hepatitis and infectious diseases; the anti-tumour usage has also been described in "Chinese Materia Medica" and "Practical Anti-Tumor Herb Medicine"[8]. *Saussurea costus* (Falc.) Lipsch is a well-known medicinal plant growing in the Himalayan region between 2500 to 3000 m above sea level. It is cultivated in a few states of India, including Uttarakhand and Himachal Pradesh. The dried roots of *Saussurea costus* have been widely used in traditional systems of medicine in Asia, as treatments for abdominal pain, tenesmus, nausea and cancer[9]. Sesquiterpene lactones such as costunolide and dehydrocostus lactone, are major components of the roots of *Saussurea costus*, and have been reported to possess various biological activities such as antineoplastic and antihepatotoxic[10,11]. *Rheum Webbianum Royle* is an important medicinal plant belonging to the family Polygonaceae. It is commonly known as 'Himalayan Rhubarb' in English, 'Ravanchini' in Hindi, 'xu mi da huang' in Chinese, 'Chotal' in Pakistan and 'Lachhu' or 'Chu-rtsa' in Ladakh. It is native to Asia-Temperate to Asia-Tropical, from China to India, Nepal and Pakistan. In India, it is found in Himachal Pradesh, Jammu Kashmir and Uttar Pradesh. The extract from the roots of *R. webbianum* are diuretic, laxative, purgative and febrifuge used against indigestion, wounds, gastritis etc[12].

A single drug cannot be effective against all types of severe liver diseases. The different plants exhibit different modes of action and in combination, that activity can be enhanced as compared to the single plant extract. Therefore, effective formulations have to be developed using indigenous medicinal plants with proper pharmacological experiments and clinical trials[13]. Also, plants in combination may exhibit various pharmacological actions like synergism that can enhance the potential medicinal effects hence, further research is necessary to screen the plants in combination for their therapeutic effects. A new approach of combining allopathic and plant-based medicaments in combination is proposed and gaining popularity but the need to understand pharmacological interactions between the drugs is necessary for effective implementation. Moreover, many authors have argued that natural molecules from phyto extracts counter balance the side effects of pure therapeutic agents and therefore these extracts have better medicinal significance[14-16].

Therefore, the present research was proposed to study the hepatoprotective effect of medicinal plants namely *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) and in various combinations against DEN induced hepatotoxicity in rats.

MATERIAL AND METHODS

Identification and extraction

The plants namely *Emblica urinaria*, *Saussure acostus* and *Rheum webbianum* were collected from different regions of Himachal Pradesh and submitted to CSIR-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, Himachal Pradesh, India for identification (Fig. 1). The powdered material from each of the plants namely *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) was weighed, macerated overnight with 70% ethanol and filtered using a double-layered muslin cloth. The filtrate was concentrated over the Rotary Evaporator (Model BUCHI Rotavapor R-210) at 40^{O}C and 175 mbar vacuum pressure until a thick slurry was obtained. The slurry was then subjected to lyophilisation for 24 hrs to obtain dried powdered plant extract. The percent yield

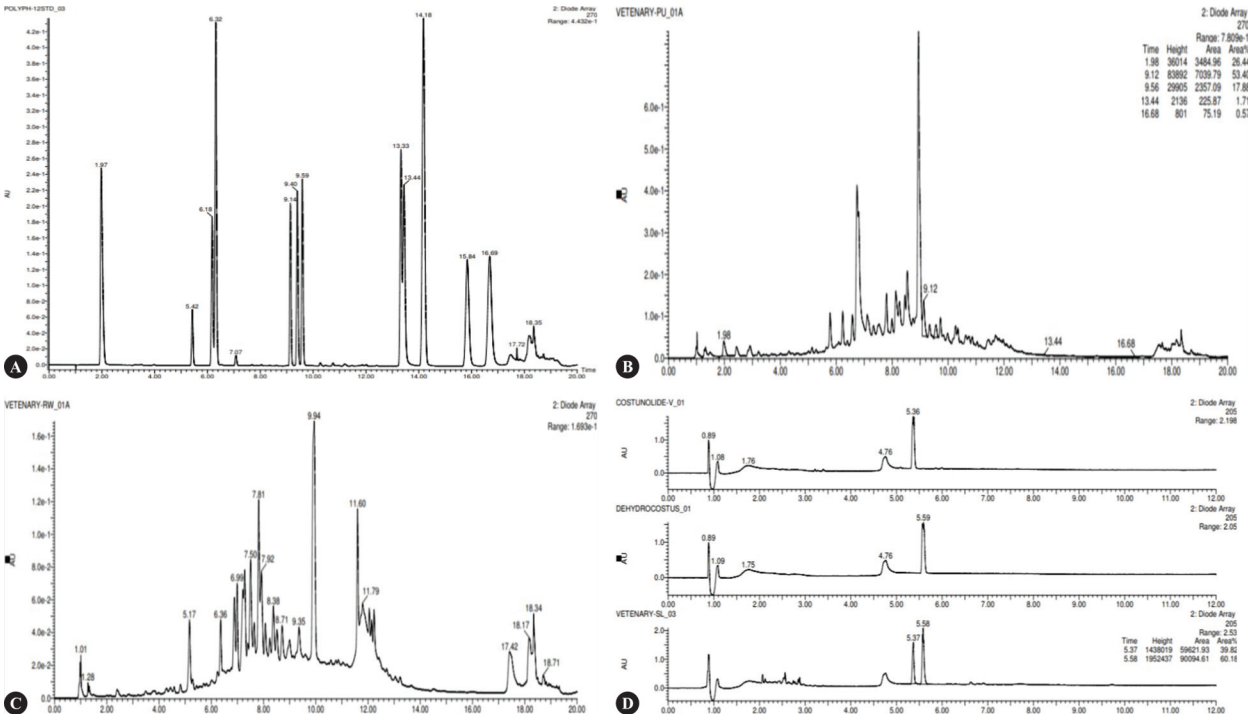

Fig. 2A. UPLC chromatogram of various standard polyphenols. **B.** UPLC chromatogram of *Emblica urinaria*. **C.** UPLC chromatogram of *Rheum webbianum*. **D.** UPLC chromatogram of standard costunolide and dehydrocostus lactone (1st and 2nd chromatogram) compared with *Saussurea costus* extract (3rd chromatogram).

was recorded. The extract was kept at 4^{O}C temperature in the refrigerator until further use.

Characterization of phytoconstituents

Qualitative and quantitative phytochemical screening of different plant extracts was done for the presence of polyphenols and their respective bioactive compounds. UPLC analysis was done to quantify the specific bioactive compounds in the extracts. Gallic acid, syringic acid, quercetin, catechin, caffeic acid, rutin, luteolin, apigenin, cinnamic acid, kaempferol, hyperoside and isoquercetin were employed as reference standards for both qualitative and quantitative analysis using Ultra-Performnace Liquid Chromatography with Diode Array Detector (UPLC-DAD) analysis. Identification of these standards on UPLC was done based on UV wavelength and retention time while the quantification was based on UV by comparing the area under the peak. UPLC columns used were Acquity UPLC-BEH-C18, 1.7 µ column of dimension 2.1x100 mm. UPLC-DAD analysis was carried out with a flow rate of 0.24 ml/min and run time was 20 minutes.

***In-vitro* studies**

The activity of the plant extracts (*Emblica urinaria*, *Saussurea costus* and *Rheum webbianum*) was subjected to *in-vitro* studies using KB (oral carcinoma), human lung and SiHa (cervical carcinoma) cells for measuring the percent cytotoxicity. For routine cell maintenance, RPMI-2640 medium containing 10% foetal bovine serum (FBS), sodium bicarbonate (2 g/L), penicillin (10,000 units/100 ml) and streptomycin (10 mg/100 ml) was used. Cells were kept at 37^{O}C in a humidified 5% CO_2 incubator. The culture media was changed frequently. Cell confluence was

Table 1. Effect of various combinations of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* extracts on the serum biochemical parameters of rats.

Groups	ALT	AST	TP	Creatinine
I	41.90 ± 0.52^{a}	106.20 ± 2.35^{a}	6.995 ± 0.09^{a}	0.70 ± 0.00^{a}
II	221.77 ± 7.50^{b}	312.14 ± 10.17^{b}	3.80 ± 0.21^{b}	0.79 ± 0.00^{b}
III	117.85 ± 2.41^{c}	150.29 ± 7.13^{c}	6.28 ± 0.14^{a}	0.70 ± 0.01^{a}
IV	184.14 ± 9.34^{d}	222.21 ± 5.30^{b}	5.85 ± 0.15^{c}	0.75 ± 0.01^{a}
V	121.476 ± 3.60^{c}	163.65 ± 9.06^{c}	6.31 ± 0.24^{a}	0.74 ± 0.01^{a}
VI	147.05 ± 12.10^{c}	181.72 ± 7.41^{c}	6.23 ± 0.44^{a}	0.72 ± 0.01^{b}

*Data represent Mean ± SE (n=6); Group I: Control group (DMSO); Group II: DEN alone; Group III: DEN + Silymarin @ 25 mg/kg bw; Group IV: DEN + plant extract combination (20 mg/kg bw *Emblica urinaria* whole plant extract + 40 mg/kg bw *Saussurea costus* root extract + 40 mg/kg bw *Rheum webbianum* root extract); Group V: DEN + plant extract combination (50 mg/kg bw *Emblica urinaria* whole plant extract + 100 mg/kg bw *Saussurea costus* root extract + 100 mg/kg bw *Rheum webbianum* root extract); Group VI: DEN + plant extract combination (100 mg/kg bw *Emblica urinaria* whole plant extract + 200 mg/kg bw *Saussurea costus* root extract + 200 mg/kg bw *Rheum webbianum* root extract); ($p \leq 0.05$)

Table 2. Effect of various combinations of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* extracts on the relative liver weight of rats.

Parameter	Groups					
	I	II	III	IV	V	VI
Relative liver weight (g)	2.28 ± 0.12[a]	3.42 ± 0.13[b]	2.77 ± 0.12[a]	3.25 ± 0.15[ab]	2.66 ± 0.02[a]	2.85 ± 0.10[a]

*Data represent Mean ± SE (n=6); Group I: Control group (DMSO); Group II: DEN alone; Group III: DEN + Silymarin @ 25 mg/kg bw; Group IV: DEN + plant extract combination (20 mg/kg bw *Emblica urinaria* whole plant extract + 40 mg/kg bw *Saussurea costus* root extract + 40 mg/kg bw *Rheum webbianum* root extract); Group V: DEN + plant extract combination (50 mg/kg bw *Emblica urinaria* whole plant extract + 100 mg/kg bw *Saussurea costus* root extract + 100 mg/kg bw *Rheum webbianum* root extract); Group VI: DEN + plant extract combination (100 mg/kg bw *Emblica urinaria* whole plant extract + 200 mg/kg bw *Saussurea costus* root extract + 200 mg/kg bw *Rheum webbianum* root extract); ($p \leq 0.05$)

examined using an inverted microscope. The cells were sub-cultured when 60-70% confluency had been attained. Cells were counted using a haemocytometer under an inverted microscope for seeding and the leftover cells were sub-cultured. *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) extracts were tested at four concentrations i.e. 20, 50, 100 and 200 µg/ml. Sulforhodamine B (SRB) assay was used to determine cytotoxicity. Optical density was measured at 540 nm through microplate reader.

Animals

The present study was conducted on 48 weaned Wistar rats of both sexes (weighing approximately 150-200 g body weight) procured from CSIR-IHBT, Palampur. Out of these 48 rats, 6 were used for the acute toxicity study, whereas 42 were used for the main experimental study. The rats were housed under strict hygienic conditions at the Laboratory Animal House facility of Dr G.C. Negi College of Veterinary and Animal Sciences, CSK HPKV, Palampur (HP) for 17 weeks. Animals were kept in polypropylene cages and rendered under standard laboratory conditions of 12 hours of light/dark cycles. The feed was autoclaved at 15 lbs pressure for 15 minutes prior to feeding to the rats. Water provided to rats was first boiled and subsequently cooled before giving to rats. Both feed and water were given *ad libitum*. During the entire period of the experiment, no medication was given to the rats. The experimental protocol was duly approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Acute toxicity study

An acute toxicity study was performed for a combination of 3 different plant extracts (*Emblica urinaria*, *Saussurea costus* and *Rheum webbianum*). Acute toxicity of extract in combination was studied in 6 Albino Wistar rats. The extract was administered orally thrice on alternate days during the first six days at the dose rate of 2 g/kg bw containing 400 mg of *Emblica urinaria*, 800 mg

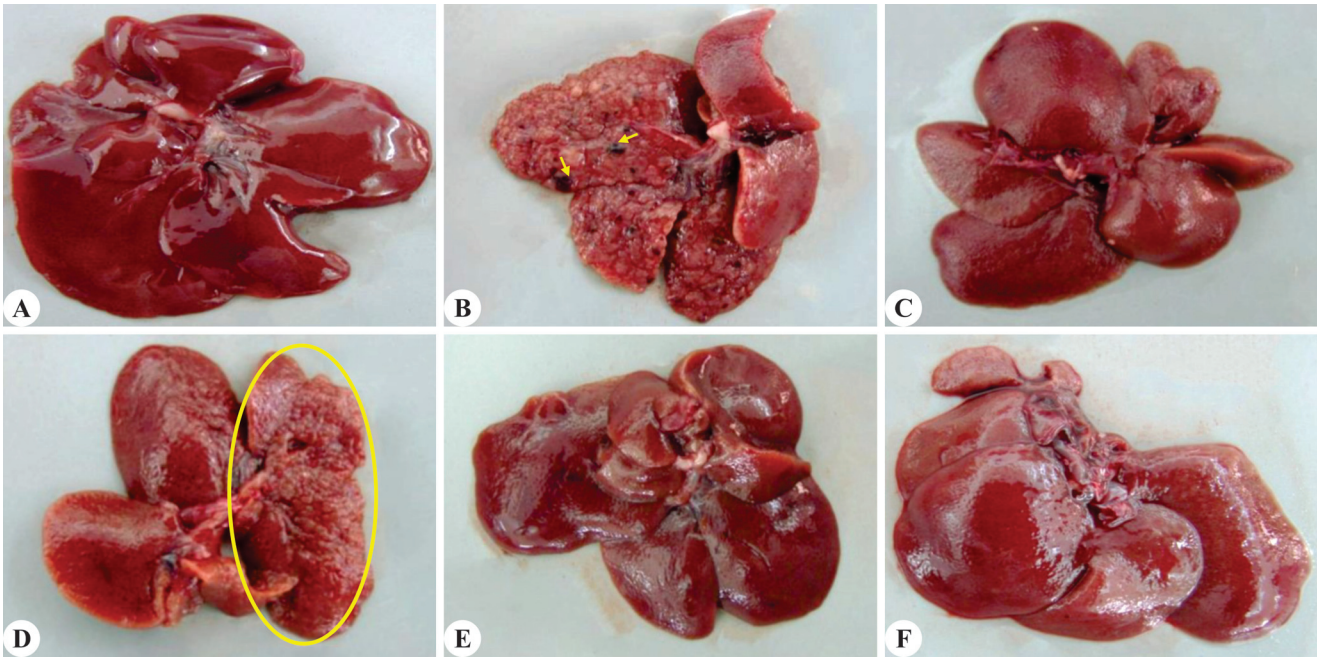

Fig. 3A. Gross pathology of normal liver (Group I). **B.** Severe multifocal to coalescing greyish-black nodules on the liver (yellow arrows) (Group II). **C.** Mild lesions present on the liver as compared to group II (Group III). **D.** Mild to moderate multifocal to coalescing greyish-black nodules on the liver (yellow circle) (Group V). **E & F.** Mild lesion present on the liver as compared to group II (Group VI).

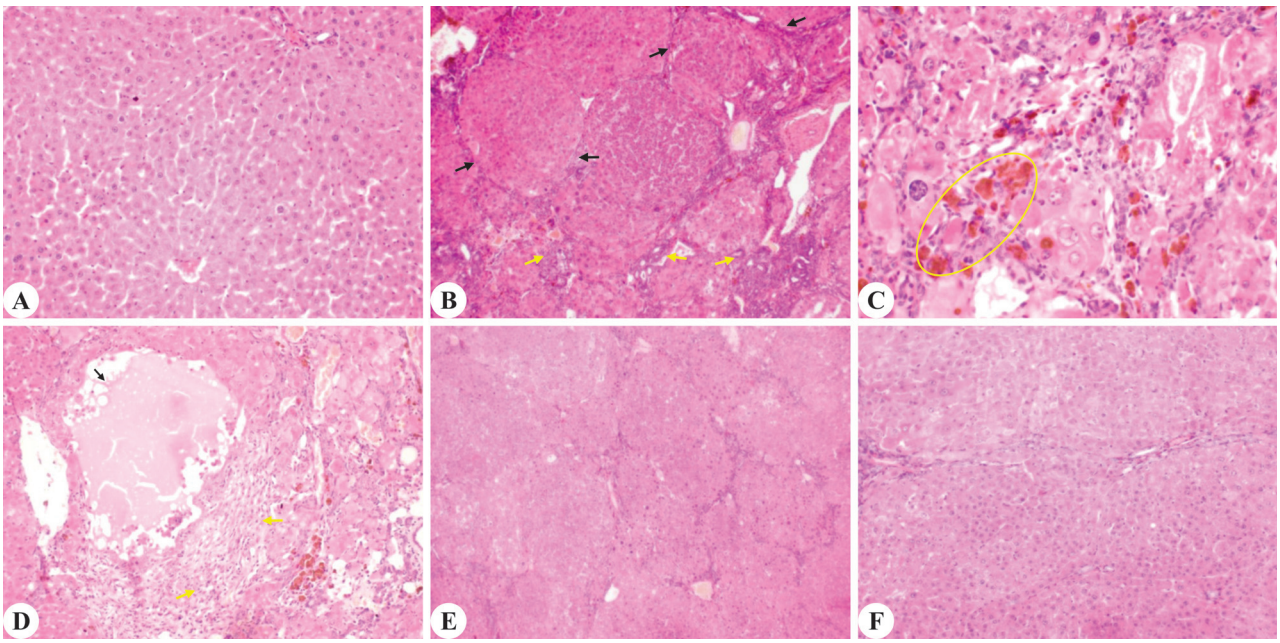

Fig. 4A. Photographs of liver showing: Normal hepatocytes and hepatic cord structure (H&E x10). **B.** Cirrhosis forming hepatocytic nodules (black arrows) and bile duct proliferation (yellow arrows) in and around the portal area (H&E x10). **C.** Apoptotic bodies, brown coloured bile accumulation in the bile duct (yellow circle) and presence of eosinophilic inclusion in the cytoplasm of the cell (H&E x20). **D.** Extensive fibrosis (yellow arrows) and bile cyst (black arrows) (H&E x10). **E.** Cirrhotic changes and presence of regenerating areas (H&E x40). **F.** Individualization of hepatocytes with maintained architecture (H&E x10).

of *Saussurea costus* and 800 mg of *Rheum webbianum*. After 14 days animals were sacrificed and examined grossly and microscopically for any toxicity.

Experimental protocol

In the present study, a total of 42 Wistar rats of either sex were randomly divided into six groups *viz.* group I served as normal control and received vehicle (DMSO) only; group II rats were treated with DEN@0.001% upto 10 weeks and thereafter the dose rate was increased to 0.01% for another 7 weeks; group III rats were treated with silymarin@25 mg/kg bw; group IV rats were treated with a combination of *Emblica urinaria*, *Saussurea costus*, *Rheum webbianum* extracts@100 mg/kg bw (1:2:2); group V rats were treated with a combination of *Emblica urinaria*, *Saussurea costus*, *Rheum webbianum* extracts@250 mg/kg bw (1:2:2) and group VI rats were treated with a combination of *Emblica urinaria*, *Saussurea costus*, *Rheum webbianum* extracts@500 mg/kg bw (1:2:2). After 10 weeks, six animals from group II were sacrificed to study the extent of liver damage on histopathological examination and the dose of DEN for the subsequent study was increased to 0.01% in groups II, III, IV, V and VI upto the end of the experiment. The doses were administered @ 0.5 ml/100g bw to the rats. The dosing of animals was done according to their daily-recorded body weights. The control animals received an equal volume of DMSO similar to those treated with the extracts.

Clinical signs, mortality pattern and growth response

The experimental animals of all the groups were observed closely throughout the experimental period thrice a day, for the development of any clinical signs and mortality. All the animals were carefully checked for the symptoms of dullness or depressed activity, reduced body weight, hair loss, skin condition and gastrointestinal symptoms. The food and water intake were monitored during the entire experiment. The body weight of all the animals was recorded every week for the calculation of dose accordingly.

Serum biochemical estimation

For serum biochemical estimation, the serum samples were collected from the animals of all the groups at the end of the experiment. Approximately 3 ml of blood was collected separately in a dry, clean and sterilized tube and allowed to clot. The sera were separated by centrifugation and further preserved at -20^{O}C till the estimations were carried out. The serum samples were analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, serum creatinine and serum total protein levels using commercially available kits (Agapee Diagnostic Ltd., India) by using Semi-automatic Biochemistry Analyzer (AGAPPE MISPA-Neo, India).

Gross pathology

At the end of the experiment, all the rats from different groups were sacrificed using CO_2 euthanasia in a closed gas chamber. Detailed necropsy examination was carried out and gross changes in the internal organs of the rats were recorded, systematically. The liver and

Table 3. Microscopic lesion scores of liver in rats from various treatment groups.

Microscopic lesions	Groups					
	I	II	III	IV	V	VI
Bile duct hyperplasia	0.00 ± 0.00^{a}	2.83 ± 0.16^{b}	1.50 ± 0.22^{ab}	2.50 ± 0.22^{b}	1.83 ± 0.30^{ab}	2.50 ± 0.22^{b}
Enhanced lobular pattern	0.00 ± 0.00^{a}	3.00 ± 0.00^{b}	0.83 ± 0.30^{ab}	1.66 ± 0.33^{b}	0.33 ± 0.21^{a}	0.83 ± 0.30^{ab}
Pleomorphism	0.00 ± 0.00^{a}	2.50 ± 0.22^{b}	1.50 ± 0.22^{ab}	2.00 ± 0.25^{b}	2.00 ± 0.25^{b}	1.83 ± 0.16^{b}
Hyperchromasia	0.00 ± 0.00^{a}	3.00 ± 0.00^{b}	1.50 ± 0.22^{a}	2.30 ± 0.21^{b}	2.00 ± 0.25^{ab}	1.66 ± 0.21^{ab}
Cirrhosis	0.00 ± 0.00^{a}	2.83 ± 0.16^{b}	1.00 ± 0.00^{a}	1.50 ± 0.22^{ab}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Bile stasis	0.00 ± 0.00^{a}	2.50 ± 0.22^{b}	1.16 ± 0.16^{ab}	2.00 ± 0.00^{b}	1.66 ± 0.33^{ab}	1.83 ± 0.30^{b}
Lipid droplets	0.00 ± 0.00^{a}	2.16 ± 0.16^{b}	1.16 ± 0.16^{ab}	1.50 ± 0.22^{b}	1.00 ± 0.00^{ab}	1.00 ± 0.00^{ab}

*Data represent Mean ± SE (n=6); Group I: Control group (DMSO); Group II: DEN alone; Group III: DEN + Silymarin @ 25 mg/kg bw; Group IV: DEN + plant extract combination (20 mg/kg bw *Emblica urinaria* whole plant extract + 40 mg/kg bw *Saussurea costus* root extract + 40 mg/kg bw *Rheum webbianum* root extract); Group V: DEN + plant extract combination (50 mg/kg bw *Emblica urinaria* whole plant extract + 100 mg/kg bw *Saussurea costus* root extract + 100 mg/kg bw *Rheum webbianum* root extract); Group VI: DEN + plant extract combination (100 mg/kg bw *Emblica urinaria* whole plant extract + 200 mg/kg bw *Saussurea costus* root extract + 200 mg/kg bw *Rheum webbianum* root extract); ($p \leq 0.05$)

spleen were weighed and the relative organ weight (weight of organ as a proportion of the total weight of each rat) was calculated.

Histopathology

After a thorough gross examination, small representative samples of approximately 5 mm thickness (liver, kidneys, spleen and intestines) were collected in 10% neutral buffered formal saline solution. After 3-4 days of fixation, the tissues were subjected to dehydration in ascending grades of ethyl alcohol, clearing in benzene, embedding with paraffin and sectioning to obtain 3-4 μ thick sections. The sections were then stained with haematoxylin and eosin (H&E) stain as per the standard protocol[17]. The bile duct hyperplasia and other degenerative changes were graded with scores as Score 1: occasional/in few (mild), Score 2: fairly common/ multifocal areas (moderate) and Score 3: diffuse (severe)[18].

Statistical analysis

The data generated during the study was analysed using One-way Analysis of Variance (ANOVA) and the results were compared by Tukey's post hoc test. All analysis was performed with Graph Pad In-Stat software (San Diago, USA). All the statements of significance were based on a probability level of P≤0.05.

RESULTS

Percent recovery of the extracts

The final yield of raw plant materials namely *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) was 5.49%, 15% and 12%, respectively.

Phytochemical screening of 70% ethanolic extracts

Various polyphenols namely gallic acid, syringic acid, quercetin, catechin, caffeic acid, rutin, luteolin, apigenin, cinnamic acid, kaempferol, hyperoside and isoquercetin were detected in *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) plant extracts (Fig. 2A). UPLC chromatograms of various standard polyphenols were obtained which were then compared with the UPLC chromatograms of *Emblica urinaria* and *Rheum webbianum* (Fig. 2B & C). Quantification of bioactive compounds of *Saussurea costus* revealed the presence of costunolide and dehydrocostus lactone at the concentrations of 3.57% and 3.68%, respectively (Fig. 2D).

Percentage (%) cytotoxicity

The % cytotoxicity of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) at the highest dose (200 μg/ml, 72 h after incubation) in human lung cancer A549 cells was 18.30, 45.82 and 9.98, respectively. The % anti-proliferative activity of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) at the highest dose (200 μg/ml, 72 h after incubation) in oral cancer KB cells was 16.94, 23.83 and 5.59, respectively. On cervical carcinoma SiHA cells % cytotoxicity of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) at the highest dose (200 μg/ml, 72 h after incubation) was found to 2.54, 6.67 and 5.73, respectively. Cytotoxicity evaluation was carried out at four concentrations (20, 50, 100 and 200 μg/ml) as described in the Materials and Methods. The concentrations of 20, 50 and 100 μg/ml produced negligible effects. Accordingly, only the data at 200 μg/ml are presented and discussed.

Clinical signs, mortality and body weight gain

The rats used in the acute toxicity study showed no clinical signs. All the rats were healthy and no weight loss was observed. Upon sacrificing no significant changes were observed grossly and histologically.

The animals in the control group i.e. group I were completely healthy with normal behaviour throughout the experiment. The rats in group II treated with DEN only showed a decrease in body weight after 12 weeks,

no level of significance was observed. The rats in group II exhibited hair loss, anorexia, lethargy behaviour and hunched posture. The severity of clinical symptoms was reduced in the ameliorative groups as compared with group II treated with DEN only. The symptoms in ameliorative groups III, IV, V and VI included roughed hair coats and were almost as normal as those of the negative control group. No mortality was recorded in any of the experimental groups, but there was a drastic decline in the body weight of animals treated with DEN only (group II) when compared to the control group (group I).

Serum biochemistry

The rats in group II treated with DEN alone showed a significant ($p \leq 0.05$) increase in the levels of ALT in comparison to the control group. Mean serum ALT activity was observed to be significantly ($p \leq 0.05$) highest in group II treated with DEN only as compared to other ameliorative groups IV, V and VI treated with different combinations of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) extracts. However, the values of serum ALT in the other treatment groups (III, IV, V, VI) were significantly ($p \leq 0.05$) elevated in comparison to the control group. The rats treated with DEN only (group II) showed a significant ($p \leq 0.05$) elevation in the mean values of serum AST levels in comparison to group I. The mean serum AST activity was observed to be significantly increased ($p \leq 0.05$) in group II treated with DEN alone as compared to the treatment groups III (DEN+silymarin), V (50 mg *Emblica urinaria* whole plant extract + 100 mg *Saussurea costus* root extract + 100 mg *Rheum webbianum* root extract) and VI (100 mg *Emblica urinaria* whole plant extract + 200 mg *Saussurea costus* root extract + 200 mg *Rheum webbianum* root extract). The groups III, IV, V and VI significantly ($p \leq 0.05$) restored the level of AST to the control group. The group treated with DEN only exhibited a significant ($p \leq 0.05$) reduction in the levels of total proteins as compared with the control group. The various combinations of plant extracts were found to be significantly ($p \leq 0.05$) effective in attenuating the level of serum total protein in comparison with the DEN-treated group. The values of serum creatinine were found to be significantly ($p \leq 0.05$) increased in group II treated with DEN only as compared to the control group i.e. group I and other treatment groups i.e. groups III, IV, V and VI (Table 1).

Effect on relative liver weight

The relative liver weight of rats treated with DEN only increased significantly ($p \leq 0.05$) as compared with the control group I. The rats in group III and ameliorative groups (V and VI) treated with different combinations of *Emblica urinaria* (whole plant) *Saussurea costus* (roots) and *Rheum webbianum* (roots) extracts exhibited significant ($p \leq 0.05$) reduction in liver weight in comparison to DEN control (Table 2).

Gross pathology

No significant gross pathological changes were evident in the liver of rats in the control group (group I) (Fig. 3A). Hepatomegaly characterized by marked enlargement of the liver with rounding of edges was a consistent gross observation in group II rats given with DEN alone. The liver surface in most of the rats in group II showed rough boundaries and shrinkage. Multiple grey to whitish coloured raised nodules ranging 1-5 mm in diameter were scattered over the entire surface of the liver (Fig. 3B). In several rats, multiple black-coloured, raised nodules were present. The rats in group III treated with silymarin exhibited a moderately rough appearance with a few coalescing nodules in comparison to DEN treated group i.e. group II (Fig. 3C). The overall appearance of the liver in group III was improved in comparison to the DEN-treated group. Groups IV, V and VI treated with different combinations of plant extracts have shown small greyish-white coloured, raised and poorly demarcated nodules over the liver surface with no evidence of black-coloured nodules (Figs. 3D & F). The sevrity of the liver damage was minimal in ameliorative groups as compared with group II rats treated with DEN only. The severity of lesions was reduced in various treatment groups and group VI was treated with *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) in the combination of 100, 200 and 200 mg/kg bw, respectively, has shown maximal protection against DEN induced liver damage.

Histopathology

The liver sections in rats in the control group i.e. group 1 showed normal hepatic parenchyma with no distortion in its hepatic cords. Hepatocytes in group I showed uniform-sized nuclei throughout the hepatic parenchyma (Fig. 4A). The rats in group II treated with DEN only showed markedly distorted hepatic cords with lobulated hepatic parenchyma. The lobular structures in the liver were surrounded by a mild to moderate degree of fibrous connective tissue. The hepatocytes within the lobule revealed varying degrees of degenerative changes which were characterized by increased cellular swelling due to eosinophilic cytoplasmic granularity. The liver section of rats in group II showed severe bile duct proliferation along with hydropic or vacuolar changes. In many of the hepatocytes, cytoplasmic details were altogether lost and appeared to contain dark homogenous eosinophilic deposits. In some places, the fibrous tissue proliferation wandered into the hepatic parenchyma which resulted in the small islands of hepatic cells. Lymphocytic infiltration along with massive deposition of golden brown pigment was consistently present in the portal areas (Fig. 4B). Microscopic examination of liver tissues in various ameliorative groups was

studied, the changes were evident in all the groups but the extent of damage was limited as compared to the positive control group i.e. group II (Figs. 4C-E). Among all the combination groups, the rats in group VI (*Emblica urinaria*:*Saussurea costus*:*Rheum webbianum*, 100:200:200 mg/kg bw) showed significantly lower lesion intensity (Fig. 4F and Table 3). The mean microscopic lesion score of changes in the liver of rats in various treatment groups is depicted in Table 3.

Lesions in the spleen were characterized by mild congestion in group II treated with DEN only. Histopathological changes in the spleen sections in group II rats revealed an increase in lymphoid pulp. The lymphoid pulp area often coalesces to form a bigger area in the splenic parenchyma. In the white pulp splenic artery seems to be displaced towards the periphery. There was a slight increase in the red pulp. Golden brown hemosider in pigment was evident. Similar changes were noticed in the combination groups (groups IV, V and VI), but the changes were less severe as compared to the group II provided with DEN only.

The examination of kidney sections in rats kept in the DEN only treated group i.e. group II exhibited obliteration in the bowman's space, swelling and severe congestion, whereas, no histopathological changes were evident in the renal tissue of the rats kept in various treatment groups. The histopathological examination of intestinal tissue of rats in group II has shown lymphocytic infiltration in the villi, hyperplasia of enterocytes and lymphoid depletion in the Peyerpatches along with fragmented nuclei. Clubbing of the villi was also evident along with the goblet cell hyperplasia. In ameliorative groups (groups IV, V, VI), the histopathological changes in the intestine section were less severe as compared to group II treated with DEN only and were comparable to group III treated with silymarin.

DISCUSSION

The different plant extracts exhibit different modes of action for curing diseases and in mixture form may exhibit enhanced activity than that of individual plants, which is known as 'synergistic action'. A particular principle in its pure form may have only a fraction of the pharmacological activity that it has in its plant matrix. This highlights the importance of using the plant as a whole or a mixture of plants for treating a disease as done in the present experimental study[19,20]. The hepatoprotective effect of the ethanolic extract of *M. azedarach* (MAE) and *P. Longum* (PLE) and their combination (BHE) against hepatic damage in rats concluded that a combination of ethanolic extract *of M. Azederach* and *P. Longum* (BHE) exerts more hepatoprotective activity as compared to the group in which these were administered separately and may act as an adjuvant in clinical conditions associated with liver damage[14]. The potential use of "SAL," a standardized blend comprised of three extracts from *Schisandrachinensis*, *Artemisia capillaries* and *Aloe barbadensis*, in mitigating chemically induced acute liver toxicities. They concluded that the composition of SAL in the ratio of 4S:8A:3L could potentially be considered as a mitigating agent for chemical induced hepatotoxicity[21].

Although the 70% ethanolic extracts of *Emblicaurinaria*, *Saussurea costus* and *Rheum webbianum* showed moderate *in vitro* activity and but their combined use exhibited quite promising hepatoprotection against DEN induced liver damge in rats. In the present study, *Emblicaurinaria*, *Saussurea costus* and *Rheum webbianum* exhibited measurable *in vitro* cytotoxic activity. Among them, *Saussurea costus* demonstrated comparatively higher effects (45.82% on A549 cells), whereas the responses of *Emblicaurinaria* and *Rheum webbianum* were modest, with particularly low cytotoxicity observed on SiHa cells (2.54% and 5.73% at 200 µg/ml, respectively). These plant extracts after *in vitro* study were used further for *in vivo* assessment of the protective effect in combination against DEN induced hepatic damage in rats. The hepatoprotective and antioxidant activity of Indian *Phyllanthus* species. The extracts were screened for hepatoprotective activity against *tert* butyl hydroperoxide (*t*-BH) induced toxicity in HepG2 cells[22]. Similarly, the induction of apoptosis by *Saussurea costus* and *Pharbitisnil* on AGS Gastric Cancer Cells. They analyzed the effects of these medicinal herbs on the proliferation and the expression of cell growth/apoptosis related molecules, by using AGS gastric cancer cells[10]. Moon and associates studied the anticancer activity of *Saussurea costus* extract by an apoptotic pathway in KB human oral cancer cells. They evaluated cell viability of KB cells by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay after treatment with 30 mg/ml of methanol extract from the dried roots of *S. costus*[23]. Another study concluded by Tabin observed that maximum aloe emodin, emodin and rhein were found in *Rheum webbianum* as compared to *Rheum spiciforme*[24]. Sun and Bu investigated the anticancer effect of emodin. They studied the effects of emodin on SGC-7901 human gastric carcinoma cell proliferation, apoptosis and regulation of phosphatase of regenerating liver-3 (PRL-3). The results showed that the proliferation of SGC-7901 cells was inhibited by emodin in a time and concentration dependent manner[25].

Diethylnitrosamine (nitrosamine) is a member of the N-nitroso compound and is considered the most important hepato toxin and carcinogen in the environment. Nitrosamine containing compounds are considered to be more effective hazards to human health as they are widely used in the industry[26]. In the liver, cytochrome P450 (CYP2E1) stimulates the N-diethylnitrosamine to form reactive oxygen species

and electrophiles. Another mechanism of nitrosamine to induce the HCC, CYP2E1 excite the Kupffer cells, which start the generation of reactive oxygen species[27]. In the present study, hair loss was evident almost in all the animals. However, the symptoms such as anorexia, hunched back posture and roughed hair coat were more pronounced in group II treated with DEN only as compared with the control and other ameliorative groups. There was a drastic decline in the body weight of animals treated with DEN only as compared to various treatment groups.

The serum ALT and AST activities were elevated, whereas, total serum protein concentration was lower in the DEN treated group as compared to the control group. The values of various serum biochemical parameters in the combination groups were generally lower as compared to group II given DEN alone, thereby suggesting the *in vivo* protective effect of the ethanolic extract of plants in combination (1:2:2) against hepatic damage. The hepatoprotective effect of *Phyllanthus Urinaria* L. on CCl_4 induced liver injury. They observed that *Phyllanthus Urinaria* treatment could reverse the increase in ALT, AST and ALP induced by CCl_4 and attenuate the pathological changes in rat liver[28]. The antihepatotoxic activity of aqueous methanolic extract of *Saussurea costus* Clarke root on D-galactosamine (D-GalN) and Lipopolysaccharide (LPS) induced hepatitis in mice. When D-GalN and LPS were co-administered resulted in higher plasma transaminase levels (ALT/AST) as compared to the control group[11].

A severe intensity of gross lesions was evident in the liver of the rats treated with DEN only. The lesions in group II rats treated with DEN only comprised hepatomegaly, liver paleness, presence of greyish-white nodules and black-coloured nodules (peliosishepatis). However, the gross lesions score intensity was lower in combination groups (groups IV, V and VI) as compared to the group administered with DEN alone (group II). Hau and co-researchers investigated that the mice, administered through an intraperitoneal route with a lethal dose of acetaminophen, followed by oral administration of *Phyllanthusurinaria* extract did not show any mortality[29]. Elsayed and associates tested the efficacy of *Saussurea costus* root extract on the reversion of already established liver fibrosis[30]. Akhtar and co-workers studied the hepatoprotective effects of *Rheumemodi* roots and their aqueous and methanolic extracts against liver damage induced by paracetamol in albino rats. The findings of our study are in connection with the studies mentioned above, where the plant extracts are found to have hepatoprotective activity[31].

Group VI (highest dose combination) showed a trend toward reduced lesion scores compared with the DEN only group; these reductions were not statistically significant for several histopathological parameters, including bile duct hyperplasia, enhanced lobular pattern, pleomorphism, hyperchromasia and bile stasis. A clear and significant hepatoprotective effect was observed only in cirrhosis, where Group VI recorded a distinctly lower score. In our study, the treatment groups exhibited marked regenerative changes and were concordant with a study conducted by[32]. The microscopic changes were also observed in the spleen, kidney and intestinal tissues of rats treated with DEN only indicating the systemic effect of DEN, although these changes were nil or minimal in other ameliorative groups given with 70% ethanolic extract of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* extracts in various combinations.

CONCLUSION

The clinical signs, mortality pattern, serum biochemistry and gross and histopathological lesions strongly suggest the promising hepatoprotective action of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* (1:2:2) in a dose dependent manner in rats. The highest dose of the combination of extracts from all 3 plants i.e. 100:200:200 mg/kg bw showed a combined hepatoprotective action against DEN induced liver damage in rats. Therefore, the present investigation warrants incorporating a combination of these plants as a medication for therapeutic purposes and needs to explore its possible mechanisms in future.

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SHORT COMMUNICATION

Serum Biochemical Alterations in Sheep Affected by Ovine Pneumonic Pasteurellosis

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ABSTRACT

Ovine Pneumonic Pasteurellosis is one of the most economically important infectious disease in sheep. The infection is caused by group of bacteria belonging to family *Pasteurellaceae*. In present study, blood samples were collected from ten (n=10) pasteurellosis affected sheep after molecular confirmation through PCR assay using 16S rRNA universal primer. Additionally, blood samples were also collected from ten (n=10) apparently healthy sheep to serve as a control group. The overall mean value of biochemical parameters *viz.* AST, ALT, calcium, phosphorus, magnesium and CPK were 96.11 ± 0.96 IU/L, 62.61 ± 2.77 IU/L, 10.99 ± 0.25 mg/dL, 7.60 ± 0.20 mg/dL, 1.90 ± 0.86 mg/dL and 9.31 ± 0.23 IU/L, respectively, which were obtained from Pasteurellosis affected sheep. Affected sheep exhibited significantly ($p<0.01$) elevated serum AST and ALT levels as compared to healthy sheep. Serum calcium and magnesium levels were significantly lower ($p<0.05$ and $p<0.01$, respectively), while phosphorus levels were significantly higher ($p<0.05$) in affected sheep. Serum CPK levels were also found higher in affected sheep although this change was non-significant. This study highlights significant alterations in serum biochemical parameters in sheep affected by Pneumonic Pasteurellosis. Elevated AST, ALT and phosphorus, along with reduced calcium and magnesium in affected sheep indicate the systemic impact of the infection. These findings contribute to understanding the patho-physiological effects of the disease and may assist in diagnostic and therapeutic approaches in pasteurellosis affected sheep.

Keywords: ALT, AST, CPK, ovine pneumonic pasteurellosis

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Sheep are highly valued in the Indian agrarian economy. They are particularly well-suited to arid and semi-arid tropical regions with marginal and sub-marginal lands. Their adaptability makes them ideal small ruminants for efficiently utilizing sparse vegetation in dryland areas through methods like rangeland management and reseeded pastures[1]. According to the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT, 2019), the leading countries in terms of sheep population are Mainland China, with 163.48 million heads, followed by India with 74.26 million heads and Australia with 65.75 million heads. India ranks second globally in sheep population, contributing over 4.03% to the global total with a recorded population of 74.26 million sheep[2]. As per the 20th Livestock Census, Rajasthan accounted for a sheep population of 7.9 million, ranking fourth in the country with a share of 10.64%.

The lungs, being in direct contact with the external environment are continuously exposed to airborne particles such as feed dust, pollutants, microorganisms and harmful chemicals, all of which can contribute to respiratory distress and disease in animals. In sheep, pneumonia represents a multifactorial syndrome, often triggered by a combination of physiological stressors and various infectious agents, making it a complex and significant respiratory condition[3]. Ovine pneumonic pasteurellosis is highly fatal infectious disease caused by group of bacteria belonging to family *Pasteurellaceae*. Bacteria belonging to this family includes *Pasteurella multocida*, *Pasteurella haemolytica* (now considered as *Mannheimia haemolytica*) and *Biberstenia trehalosi*. These are gram negative, cocco-bacilli shaped non-spore forming bacteria. High morbidity and mortality caused by the organism result in significant economic losses among sheep farmers in arid and semi-arid region of Rajasthan.

During the septicemic stage of ovine pneumonic pasteurellosis, disruptions in serum biochemical parameters may impose physiological stress on affected animals, ultimately contributing to decreased productivity. Given the sparseness of literature addressing the impact of pasteurellosis on the serum biochemical profile of sheep, the current investigation was undertaken to evaluate these alterations in naturally infected sheep with *Pasteurella* spp.

Table 1. Detail of 16s rRNA primer gene for identification of *Pasteurella* spp.

Species (Target gene)	Primer Sequence (5′-3′)	Annealing Temperature	Amplicon Size (bp)	Reference
Pasteurella spp. Universal primer (*16s rRNA* gene)	Fp AGAGTTTGATCMTGGCTCAG Rp CGGTTACCTTGTTACGACTT	52^{O}C	1466	Lane (1991)[18]

A cross-sectional study was carried out under field conditions, wherein all animals were maintained within the same geographical region and subjected to uniform climatic and environmental factors. Study population consist of sheep suffering from pneumonia irrespective of their age, sex and breed. Also, there was no history of vaccination among the herd. The study was conducted from July 2024 to December 2024. The research on live sheep was conducted in accordance with the guidelines and with the prior approval of the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India vide letter no. CVAS/IAEC/2024-25/41.

Blood samples and nasal swab samples were collected aseptically from ten (n=10) affected sheep. Additionally, blood samples were also collected from ten (n=10) apparently healthy sheep to serve as control. Blood samples were collected by jugular venipuncture and collected in non-EDTA vials for serum separation. Serum samples were separated by making blood slant and incubated for 1 hour at 37^{O}C. Blood clots were broken and tubes were centrifuged at 2500 rpm for 30 minutes. Serum samples were pipetted out in small Pyrex tubes and were stored at -20^{O}C in deep freeze till biochemical parameters assessment.

All the nasal swab samples were individually processed. Isolation and purification of DNA was performed using HiPurA® Multi-Sample DNA Purification Kit, HiMedia (Maharashtra, India) according to the manufacturer's instructions by spin column protocol.

16S rRNA universal primer was selected for identification of *Pasteurella* spp. Primer was synthesized by Bioserve Biotechnologies (India) Private Limited, Hyderabad, India. Detail of primer is mentioned in Table 1.

Molecular identification of the target genes was conducted through PCR amplification using the Sure Cycler 8800 (Agilent Technologies, California, USA) and universal primer. PCR conditions, including the annealing temperature for gene was optimized during the initial standardization process. The PCR amplification reaction consisted of 2 µL of DNA template combined with 18 µL of reaction mixture, which included 7 µL of nuclease free water, 0.005 µM of each forward and reverse primer and 10 µL of 2X PCR Master Mix (Hi-Chrom PCR REDy Master Mix, Himedia, Maharashtra, India).

For serum biochemical examination, serum samples were analyzed for different biochemical parameters *viz.* Alanine amino transferase (ALT), Aspartate amino transferase (AST), Creatinine Phosphokinase (CPK), Calcium, Phosphorus and Magnesium by using commercial kit of 'Meril Diagnostics Pvt. Ltd.' in Spectra Lab Genie Semi-Automatic Biochemistry Analyzer.

The data obtained from both apparently healthy and Pasteurellosis affected sheep were analyzed using Software Package for Social Sciences (SPSS) Version 27.0 (IBM Corp., 2020) with significance level taken at $p \leq 0.05$ and $p \leq 0.01$.

Mean values of biochemical parameters *i.e.* serum AST, ALT, CPK, calcium, phosphorus and magnesium of Pasteurellosis affected and clinically healthy sheep are presented in Table 2.

In sheep that were found affected by pasteurellosis, overall mean serum levels of AST and ALT were significantly ($p<0.01$) elevated compared to those in clinically healthy sheep. This increase in serum AST and ALT levels may be attributed to hepatic inflammatory, degenerative and necrotic changes induced by the bacterial infection and its associated circulating toxins[4-9]. The increased serum AST activity observed in

Table 2. Values of serum biochemical parameters of clinically healthy and Pasteurellosis affected sheep (Mean±SE).

S.No.	Parameters (Units)	Clinically Healthy Sheep	Pasteurellosis Affected Sheep
1.	Serum AST/SGOT (IU/L)	78.17 ± 1.35	96.11 ± 0.96**
2.	Serum ALT/SGPT (IU/L)	24.48 ± 1.28	62.61 ± 2.77**
3.	Serum Calcium (mg/dL)	12.06 ± 0.27	10.99 ± 0.25*
4.	Serum Phosphorus (mg/dL)	6.39 ± 0.29	7.60 ± 0.20*
5.	Serum Magnesium (mg/dL)	2.54 ± 0.11	1.90 ± 0.86**
6.	Serum Creatinine Phosphokinase (IU/L)	9.12 ± 0.31	9.31 ± 0.23^{NS}

** : Highly Significant at ($p<0.01$); * : Significant at ($p<0.05$); NS : Non-Significant

affected sheep may also be attributed to reduced feed intake and starvation, as well as to its muscle origin, resulting from dystrophic muscle damage associated with prolonged recumbency or increased respiratory muscle exertion in severe cases of disease[10-12]. These alterations are indicative of systemic involvement and underlying metabolic imbalances frequently linked to infections caused by *Pasteurella multocida* and *Mannheimia haemolytica* infections. The observations of the present study regarding serum AST and ALT levels are consistent with previous reports. El-Latif and El-Dessouky[4] documented mean serum AST activities of 38.67 ± 1.04 IU/L in clinically healthy lambs and 43.04 ± 0.90 IU/L in lambs with respiratory disorders, along with corresponding ALT values of 24.73 ± 0.98 IU/L and 29.86 ± 1.09 IU/L, respectively. Comparable observations have been reported in bovines[10], with mean serum AST activities of 1.1 ± 0.4 µkat/L in healthy calves and 2.1 ± 1.4 µkat/L in diseased calves. Another report[6] recorded AST values of 68.1 ± 0.4 IU/L in healthy bovines and 71.16 ± 0.69 IU/L in affected animals.

A highly significant ($p<0.01$) effect was observed in serum magnesium levels and a significant ($p<0.05$) effect were noted in serum calcium and phosphorus levels in sheep affected by pasteurellosis. The overall mean serum calcium and serum magnesium levels were lower, while serum phosphorus levels were higher in affected sheep compared to clinically healthy controls. The decreased calcium and magnesium levels may be attributed to anorexia and impaired intestinal absorption associated with the disease. Altered mineral profiles, particularly hypocalcemia and hypomagnesemia may be linked to anorexia, intestinal malabsorption and systemic stress[13]. Hyperphosphatemia, often seen in inflammatory and renal dysfunction, highlights the reciprocal regulatory relationship between calcium and phosphorus wherein hyperphosphatemia leads to a reduction in serum ionized calcium through mass action interactions between phosphate and calcium ions[14]. This imbalance is further exacerbated by impaired renal synthesis of 1, 25-dihydroxyvitamin D, which plays a crucial role in maintaining calcium homeostasis[15]. The present findings on serum calcium, magnesium and phosphorus levels are in agreement with earlier studies. El-Latif and El-Dessouky[4] reported mean serum calcium concentrations of 12.06 ± 0.27 mg/dL in clinically healthy lambs and 10.99 ± 0.25 mg/dL in affected lambs. Similarly, another finding[15] observed mean serum calcium levels of 8.643 ± 0.601 mg/dL in healthy goats and 7.280 ± 0.234 mg/dL in infected goats. They also recorded mean serum magnesium concentrations of 2.473 ± 0.387 mg/dL in healthy goats compared to 1.347 ± 0.167 mg/dL in infected goats, along with mean serum phosphorus levels of 6.213 ± 0.854 mg/dL in healthy goats and 4.173 ± 0.187 mg/dL in infected goats.

The overall mean serum CPK level was non-significantly higher in affected sheep compared to the mean values observed in clinically healthy sheep suggesting sub-clinical muscular damage or stress-related myopathy, as previously reported in severe cases involving respiratory distress and prolonged recumbency[10-12,16,17].

CONCLUSIONS

A comparative serum biochemical analysis between pasteurellosis affected sheep and clinically healthy controls revealed notable differences, indicative of systemic involvement. Affected sheep showed significantly elevated levels of serum AST, ALT and phosphorus, alongside significantly reduced serum calcium and magnesium concentrations. Additionally, a non-significant increase in serum CPK levels was observed. These biochemical changes suggest multi-organ dysfunction and metabolic disturbances often associated with bacterial infections such as those caused by Pasteurella multocida and Mannheimia haemolytica. These findings highlight the systemic nature of pasteurellosis and targeted therapeutic strategies. The incorporation of supportive mineral supplementation, prompt administration of appropriate antibiotic therapy and the implementation of effective vaccination strategies can collectively aid in reducing the adverse effects of the disease, thereby enhancing animal health and overall productivity in sheep production systems.

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SHORT COMMUNICATION

A case of Cholangio-cellular Carcinoma in Sptiz Dog

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ABSTRACT

A 12-year-old female spitz dog was brought for necropsy to the Department of Veterinary Pathology, Ranchi College of Veterinary Science and Animal Husbandry. On gross examination, liver showed multifocal white hard nodular growths ranging from 2 mm to 1.5 cm in diameter. Both lungs showed few nodular lesions in diaphragmatic lobe. On histopathological examination, liver parenchyma showed presence of anaplastic cells distributed in lobular and multifocal ductal structures suggestive of cholangiocellular carcinoma. There was marked proliferation of fibrous connective tissue in portal area, suggesting initiation of cirrhotic changes. Microscopically, metastatic lesion of cholangiocarcinoma was observed in heart. Significant metastatic changes and venous tumor thromboemboli were also observed in lungs. Simultaneous occurrence of cholangiocellular carcinoma along with metastasis in heart is a rare condition.

Keywords: Cholangiocellular carcinoma, cirrhosis, heart, metastasis, thromboemboli

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Incidence of cholangiocellular carcinoma (CCC) along with metastasis in heart is quite rare in canines. Cholangiocellular carcinoma (CCC) is an aggressive neoplasm arising from either the extrahepatic or intrahepatic biliary tree[1]. CCC is reported as the second most common liver tumour, accounting for all malignant liver tumours[2]. The distribution patterns of cholangiocellular carcinoma in dogs are classified as massive, nodular (multiple) and diffuse forms, was reported[2]. The rate of extrahepatic metastasis has been reported to be as high as 88%, with metastatic foci frequently developing in the lungs, regional lymph nodes and peritoneal cavity[3].

A 12-year-old female spitz dog was brought for necropsy to the Department of Veterinary Pathology, Ranchi College of Veterinary Science & Animal Husbandry with a history of non-specific clinical signs like anorexia, weight loss, azotemia and right hind limb lameness.

Hematological examination of the dog was suggestive of anemia with hemoglobin level of 6 g/dl and lowered packed cell volume (20%). Neutrophilic leucocytosis was observed with Total Leukocyte Count of 31200/mm^3 and 84% neutrophils with absolute neutrophil count 26,208/cm. Biochemical examination revealed elevated Alkaline Phosphatase (ALP) 1267 U/L, Aspartate Transaminase (AST) 93.56 U/L, Alanine Transaminase (ALT) 425I U/ml. Azotemia was evident with elevated blood urea (153.59 mg/dl) and serum creatinine (3.84 mg/dl), indicative of hepato-renal dysfunction.

Most cases of CCC in canines have documented elevations in serum ALP, AST and ALT activities. Less common findings during CCC include hypoalbuminemia, hyperbilirubinemia and elevated serum bile acids, which is suggestive of decreased hepatic function and/or post-hepatic cholestasis[1].

Necropsy revealed poor body condition with generalized reduction in muscle mass. On external examination, the visible mucous membranes were icteric. All organs were critically examined and tissues were collected in 10% Neutral Buffered Formalin (NBF), processed routinely and embedded in paraffin. Paraffin sections of 3-5 µm thickness were routinely cut in rotary microtome and stained with Hematoxylin and Eosin.

Grossly, liver appeared hard in consistency with multifocal white nodular growths ranging from 2 mm to 1.5 cm in diameter. At places, large confluent lesions were noticed (Fig. 1). Histopathology of tumor lesion in the liver revealed the presence of anaplastic cells distributed in a lobular pattern with interspersed fibrous connective tissue along with presence of multifocal ductal structures (Fig. 2). The lining epithelial cells of ductular structures manifested atypical changes suggestive of cholangiocellular carcinoma. In addition, marked proliferation of fibrous connective tissue was observed in portal area, suggesting initiation of cirrhotic changes. Anaplastic/atypical changes were characterized by proliferating neoplastic cells of

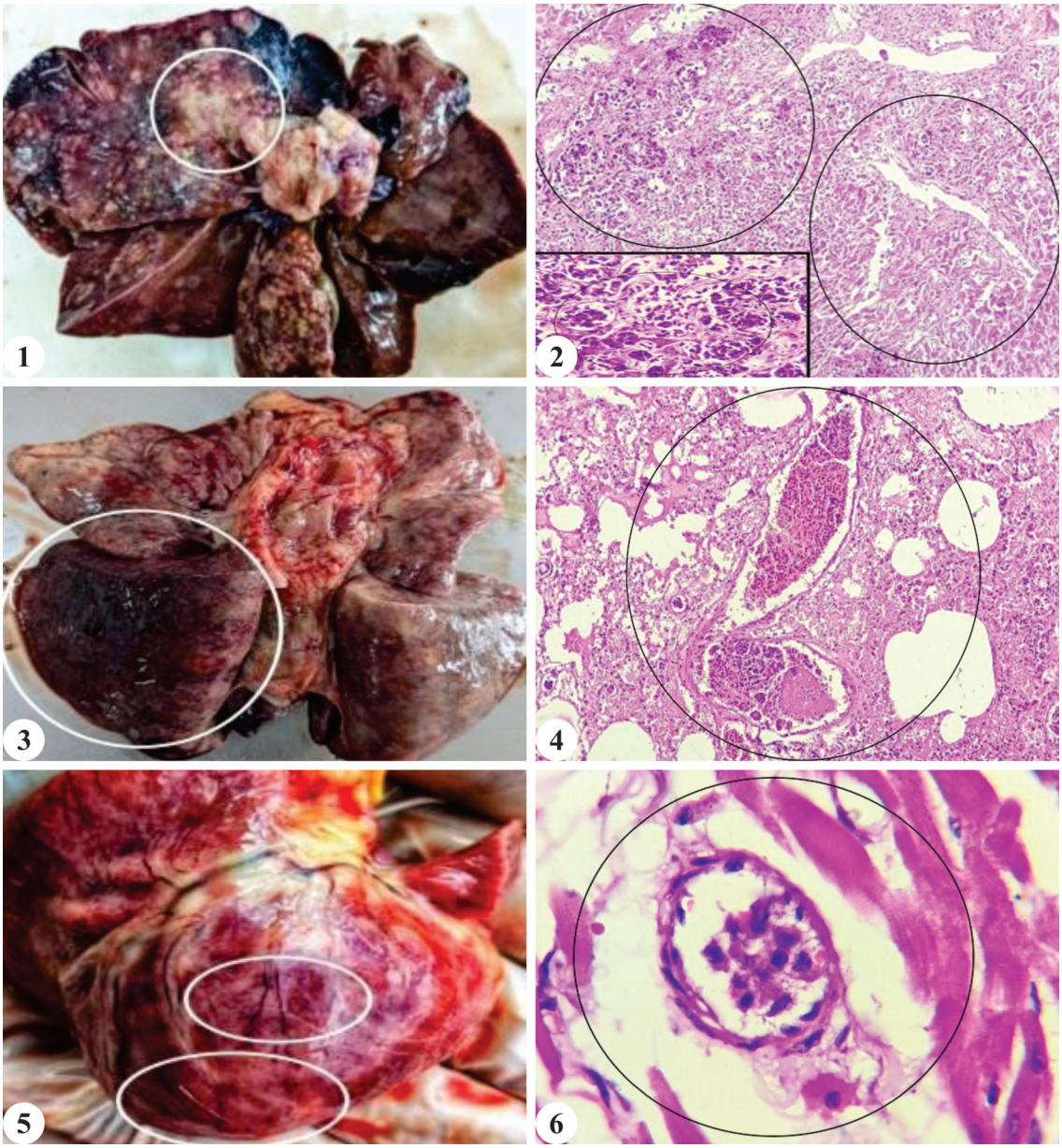

Fig. 1. Large confluent lesion in the liver; **Fig. 2.** Liver (Left circle) - Proliferating neoplastic cells of CCC with vague ductular morphology (H&E x100), (Right circle) - Atrophy of hepatocyte with dilated sinusoid space (H&E x100) and Rectangular box (Inner circle) - Pocket of neoplastic cell of Cholangiocellular carcinoma (H&E x400); **Fig. 3.** Lungs grey-red - Mottled appearance with neoplastic lesion, especially in the diaphragmatic lobe; **Fig. 4.** Lungs - Cells showing metastatic changes and venous tumor thromboemboli (H&E x100); **Fig. 5.** Rounding appearance of heart with mottled appearance; **Fig. 6.** Heart - Metastatic tumor (H&E x1000).

CCC with vague ductular morphology. Malignant bile duct epithelium showing disruption and invasion of adjacent hepatocytes. CCC has previously been reported to consist of widespread multinodular, umbilicated masses with a firm texture due to extensive necrosis and an intense scirrhous or desmoplastic response[1]. Lungs showed mottled appearance with few prominent nodular lesions in diaphragmatic lobe (Fig. 3). Histopathology of lungs also showed metastatic changes along with venous tumor thromboemboli observed in lungs with infiltrating tumor cells in the lungs interstitium suggestive of metastatic changes along with marked interstitial pneumonia and alveolar emphysematous changes (Fig. 4). Most of the blood vessels in visceral organs were packed with leukocytes. Tumor emboli in the dilated vein with seeding of tumor cells (malignant cholangiocytes) with glandular differentiation in the lung parenchyma. Marked hemorrhage in lung parenchyma with highly congested and dilated alveolar capillary. Significant fibrous exudate was observed in alveolar space. Cholangiocytes in lung parenchyma revealed atypical cellular changes. Significant eosinophilic

infiltration observed on alveolar space alongside mononuclear cell infiltration. Focal area of metastatic tumor cell proliferation also observed.

Heart was dilated having rounded appearance, mild hemorrhages were present on the epicardium (Fig. 5). Myocardium was pale in appearance. Histopathologically, a foci of metastatic cholangiocellular carcinoma in heart was observed which is a rare site for metastasis (Fig. 6) degenerative and infiltrative changes were observed. Grossly, the mucosal surface of stomach showed hemorrhages with presence of multifocal ulcers measuring about 1-2 mm in diameter. Sub adventitia revealed hemorrhage and marked congestion of anterior vein and capillaries. Villi epithelium showed degenerative changes and cloudy swelling along with necrosis. Fusion of entire length of villi giving it a continues look. Blood vessels of lamina propria were dilated and congested. Crypt showed necrosis and degeneration. Kidney appeared shrunken with irregular surface and adhesion of capsule. Capsule peeled off easily. Histopathologically, kidney showed marked necrotic and degenerative changes of renal epithelial cells. Segmental degenerative changes were also observed in glomeruli. Nephrosclerotic changes with interstitial fibrosis was evident. Spleen showed the presence of multiple red infarcts on the margins. Microscopically, spleen had mononuclear cell infiltration many of which showed deposits of hemosiderin. Histological changes in the liver were suggestive of frank cancer of CCC with metastatis in lungs and heart.

Dog was anemic due to neoplastic condition. It is anemia of chronic inflammation due to persistent immune stimulation and inflammatory cytokines (ex.IL-6) inhibiting erythropoiesis[5]. Also, GI ulcers seen in stomach may have led to chronic blood loss. Enlarged spleen with infarct and hemosiderin deposit suggests increased red cell destruction or polling. Paraneoplastic leukocytosis can produce cytokines like G-CSF (granulocyte colony stimulating factor) stimulating neutrophil production[4]. Extensive tumor necrosis and tissue damage instigate an inflammatory response. Liver tumor disrupts hepatocellular and biliary architecture releasing enzymes. ALP is significantly elevated in biliary obstruction and cholestasis (prominent in CCC). ALT/AST was elevated due to hepatocellular injury or necrosis. Ductular proliferation and fibrosis may impair bile flow. Cancer-related cachexia and anorexia, lead to prerenal azotemia[5].

Biliary epithelial cells undergo genetic and epigenetic alterations in regulatory genes, which accumulate and lead to the activation of oncogenes and the dysregulation of tumor suppressor genes (TSGs)[6]. Malignant transformation of cholangiocytes arises against a background of chronic inflammation of liver which causes repeated cycles of repair and damage which increases the likelihood of mutation in genes like KRAS and P53[7]. Damaged cells release cytokines like IL-6, TNF-alpha, which activate the immune system, causing chronic inflammation. IL-6 increases expression of progranulin, a precursor protein for granulins (a family of peptides that regulate cell growth) resulting in activation of the PI3K (phosphoinositide 3-kinase) pathway which mediates cell survival, mitosis, migration and angiogenesis[8].

TGF-beta from damaged cells, activate hepatic stellate cells present in the space of Disse, which differentiate into myofibroblast, produce extracellular matrix and cause fibrosis[9]. Fibrosis along with chronic inflammation causes cellular hypoxia. Bile duct epithelium undergoes dysplasia, decrease E- Cadherin, cause motility of tumor cells and causes invasion. Hypoxia inducible factor-1alpha (HIF-1alpha) release vascular endothelial growth factor (VEGF) which further causes angiogenesis[10]. Metastasis along with tumor causes systemic inflammation, multi-organ failure, cardio-respiratory failure and finally death.

This case adds to the limited veterinary literature on cholangiocarcinoma (CCC) in canines, especially regarding its metastatic patterns and histopathology.

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SHORT COMMUNICATION

A case of invasive mammary carcinoma in a female dog: Histological and immunohistochemical study

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ABSTRACT

Mammary tumours are the most frequently found neoplasm in unspayed female dogs and most of them exhibit malignant characteristics. This case report presents a 14-year-old female dog with an ulcerated mass in the left caudal inguinal mammary gland. Clinical examination, diagnostic imaging, cytology and histopathological assessment confirmed the neoplasm as invasive mammary carcinoma. Histopathological examination revealed absence of tubule formation, high-grade nuclear atypia and frequent mitoses consistent with aggressive morphology. Neoplastic epithelial cells showed invasion into the surrounding connective tissue stroma forming varying numbers of cellular aggregates. This case emphasizes the importance of early diagnosis and comprehensive histomorphological assessment for determining prognosis and treatment strategy.

Keywords: Dog, histological, immunohistochemical, mammary carcinoma

Canine mammary tumours (CMTs) comprise the most prevalent neoplasm observed in female dogs encompassing nearly half of all mammary gland tumours[1]. A wide range of biological spectrum can be displayed by these neoplasms varying from benign forms to invasive malignant forms. Hormonal factors specially estrogen and progesterone play a pivotal role in development of these tumors. Early spaying significantly reduces the risk of mammary tumour development underscoring the hormonal dependency of mammary tumor genesis in canines[2]. Several mammary neoplasms exhibit malignant behaviour involving local invasion and distant metastasis. Invasive mammary carcinoma constitutes a remarkable clinical challenge due to its aggressive behaviour, poor prognosis and chances of recurrence. CMTs show similarities to human breast cancers making them useful models for comparative oncology. This resemblance has prompted interest in using canine neoplasia to understand the tumour biology and to develop new therapeutics[3]. This report presents a case of invasive mammary carcinoma in a female dog, emphasizing the clinical presentation, diagnostic findings and histopathology.

A 14-years-old non-descript female dog was presented to the Referral Veterinary Polyclinic and Teaching Veterinary Complex, ICAR-IVRI, Izatnagar, Uttar Pradesh with a history of progressively developed swelling in the left caudal inguinal mammary gland since one and a half months. On clinical examination, an ulcerating, exudating, soft to firm and pedunculated mass measuring 2.3x2.1 cm was observed (Fig. 1). The physiological parameters of the dog were within the normal range. Fine needle aspirate from the tumour mass was taken and stained using Giemsa stain. Thoracic radiograph and ultrasonography of abdominal region was done to detect presence of metastatic lesions. Surgical excision of the mass was done and the tissue was preserved in 10% neutral buffered formalin (NBF). Histopathological evaluation of the section was done after haematoxylin and eosin (H&E) staining. Grading was done based on tubule formation, nuclear pleomorphism and mitosis per ten high power field (hpf)[4].

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Metastasis to lungs was not observed in the thoracic radiograph. Ultrasonography revealed the presence of an ill-defined, heterogenous lesion measuring 13x8 mm in spleen (Fig. 2). Giemsa stain of fine needle aspirate collected from mammary gland tumour revealed loosely cohesive cluster of neoplastic cells with indistinct cell borders. Pleomorphic nuclei were observed with coarse chromatin, vacuolations and prominent nucleoli (Fig. 3). Histopathological evaluation revealed infiltrative nests of tumor cells forming lobular patterns and infiltrated into the stroma (Fig. 4a-c). The neoplastic cells were identified either as individual cells or arranged in

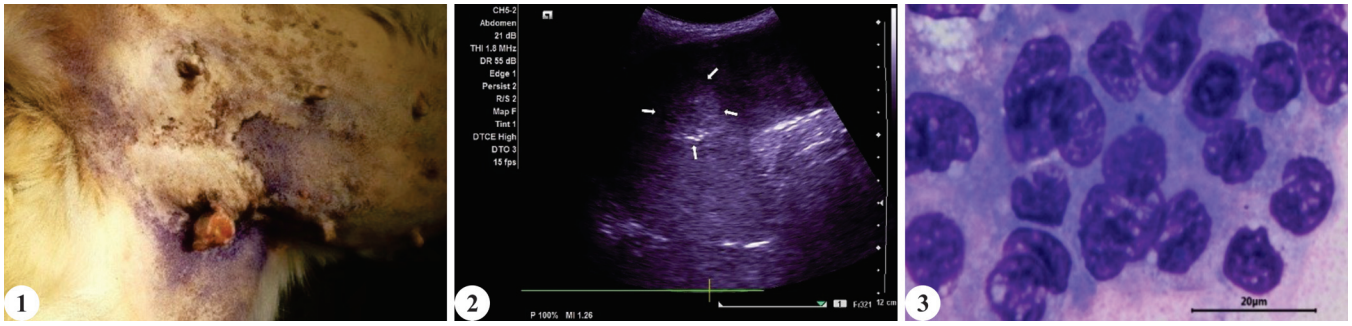

Fig. 1. Dog showing nodular ulcerated tumour growth with irregular margins in left inguinal mammary gland; **Fig. 2.** Ultrasonograph showing an ill defined lesion measuring 13*8 cm in spleen; **Fig. 3.** Cytological smear revealing cluster of neoplastic cells with highly pleomorphic nuclei, coarse chromatin with nuclear vacuolations and prominent nucleoli (Giemsa x1000).

irregularly distributed tumour islands within the stromal component, lacking tubule formation and exhibiting high-grade nuclear atypia. Mononuclear inflammatory cells were noted surrounding the neoplastic cells. The neoplastic epithelial cells were characterized by indistinct cytoplasmic boundaries and marked pleomorphism, with frequent mitotic figures. Connective tissue proliferation was evident in the mesenchymal component. Based on these findings the tumour was categorized as a high-grade tumour. Histological grading remains a significant prognostic factor, with higher grades correlating with increased aggressiveness and poorer outcomes[5].

Immunohistochemistry was performed for estrogen receptor (ER) and progesterone receptor (PR). Nuclear immunoreactivity for ER was not observed in neoplastic cells (Fig. 5a). Nuclear immunoreactivity for progesterone receptor (PR) was detected in approximately 30% of neoplastic cells with moderate staining intensity, indicating partial hormone receptor positivity (Fig. 5b). Canine invasive mammary carcinomas present diverse histological types, including solid, tubular, lobular, papillary and adenosquamous types. These tumors are characterized by infiltrative growth into surrounding stromal tissues, presence of neoplastic epithelial cells with varying degrees of pleomorphism and frequent mitotic figures. The stromal response often includes mononuclear inflammatory cell infiltration and connective tissue proliferation[6].

Histological grading remains a significant prognostic factor with higher grades correlating with increased aggressiveness and poor outcomes[5]. A remarkable reduction in ER expression has been observed as tumour progresses to malignant types particularly invasive carcinoma[3]. ER-positive canine mammary carcinomas are often associated with better prognosis, including lower histological grade and longer survival times[7]. The presence of hormone receptors like ER and PR may also suggest the potential responsiveness of these tumors to hormonal therapy[8,9,10]. Normal and benign neoplasms express both ER and PR[11], whereas low ER expression is associated with worse prognosis in malignant mammary neoplasms[12].

The present case emphasizes the aggressive behaviour of canine invasive mammary neoplasms distinguished by high histological grade, marked cellular pleomorphism and negative ER estimation. Hormone dependent phenotype is reflected by the negative ER expression status along with partial PR positivity indicating poor prognosis and reduced response to therapy. The loss of ER expression is associated with high grade malignant mammary neoplasms and correlate with increased invasiveness and metastatic potential. These observations underscore the importance of histopathological and immunohistochemical profiling for

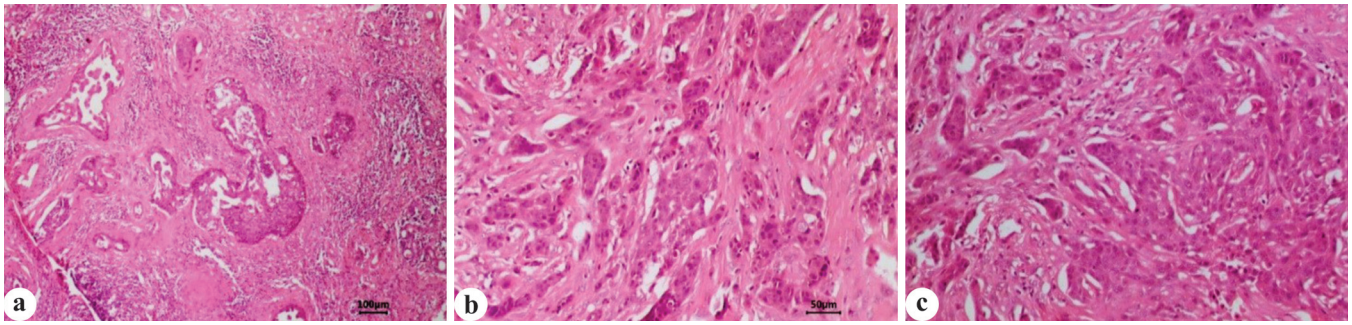

Fig. 4a. Infiltrative nests of tumor cells forming lobular patterns surrounded by inflammatory cells (H&E x100). **b & c.** Neoplastic epithelial cells either singly or forming tumour islands, distributed irregularly in stromal component without tubule formation with high grade nuclear atypia, marked pleomorphism with frequent mitotic figures (H&E x200).

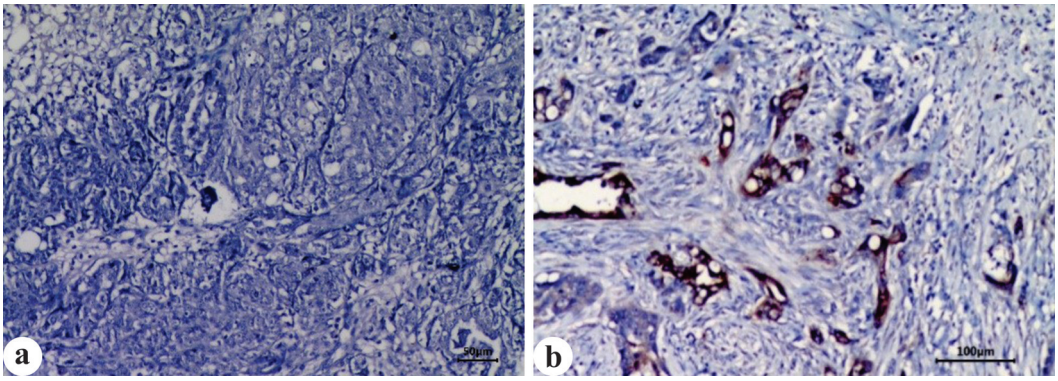

Fig. 5a. Nests of epithelial cells showing bluish hematoxylin counter stain only in tissue sections immunostained for ER. **b.** Moderate nuclear immunostaining for PR in the epithelial cells (DAB x Meyer's hematoxylin x100).

neoplasms classification as well as to define the prognosis of the case and personalized therapeutic strategies. The dog died after three months of surgical excision of the tumour.

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Use of Artificial Intelligence (AI)-Assisted Technology for manuscript preparation: The authors confirm that there was no use of AI-assisted technology for assisting in the writing of the manuscript and no images were manipulated using AI.

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SHORT COMMUNICATION

Oral melanoma with multicentric metastasis in a sniffer dog

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ABSTRACT

A twelve-year-old airport sniffer male Labrador dog was brought to Veterinary Clinical Complex, VCRI, Namakkal with the history of sudden seizures and drooling of saliva. However, the dog was found dead while on arrival at the Veterinary Clinical Complex. The dog carcass was referred to the Department of Veterinary Pathology, VCRI, Namakkal for necropsy. On gross examination, multiple blackish nodular growths in the oral cavity; leathery appearance of lungs with tiny raised greyish nodules; dark blackish raised nodules in spleen; shrunken kidneys with focal blackish areas in cortico-medullary junction; blackish discolouration with haemorrhage in intestine; thickened urinary bladder and prostate gland were observed. Cytological examination of oral growth revealed clusters of melanin-laden melanocytes with anisocytosis, anisokaryosis, prominent nucleoli and intracytoplasmic coarse granular melanin pigments. Histopathological examination of the oral growth exhibited replacement of normal stratified squamous epithelium with numerous melanin-laden melanocytes. In addition, melanin-laden melanocytes were also noticed in various organs like lungs, spleen, kidney and intestine. This case deals with oral melanoma with multicentric metastasis to various organs in a sniffer dog.

Keywords: Melanoma, metastasis, multicentric, sniffer dog

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Oral melanoma represents the most prevalent and aggressive oral neoplasm in dogs[1,2]. It usually arises from the mucocutaneous junction of lips, gums as well as from any part of the oral cavity[3]. Its incidence increases with age[4] and most commonly above 10 years of age[5]. Low grade malignant oral melanomas are common in 8 years of age, whereas high grade malignant forms are more frequent in 12 years of age[6]. Most of the malignant melanomas are fatal in life as it grows very rapidly and metastasis to various organs. It can spread mainly through lymphatics as well as regional lymph nodes and thereafter, it spreads to lungs and other organs like heart, brain, spleen and gastrointestinal tract[3,7,8]. Melanomas are the most common tumours which originate from the melanoblast and melanocytes of neuroectodermal origin[9]. Various causes for malignant melanoma include trauma, hormones, chemical exposure and genetic susceptibility[10]. Because of its aggressiveness and its tendency to spread to lungs, canine oral melanoma is considered as valuable model for developing novel therapies in humans[11]. Recent studies have shown that oral melanoma is highly helpful in finding novel protocols for immunotherapy especially in development of modified vaccines for both primary and metastatic neoplastic conditions[12]. This case report deals with oral melanoma and its propensity for multicentric metastasis in a sniffer dog.

A twelve-year-old sniffer male Labrador was brought for treatment to Veterinary Clinical Complex, VCRI, Namakkal with the history of sudden episodes of seizures and drooling of saliva. However, the dog was found dead while on arrival at the clinical complex. The carcass was referred to the Department of Veterinary Pathology, VCRI, Namakkal for necropsy. A detailed postmortem examination was carried out and gross lesions were recorded. Fine needle aspiration cytology (FNAC) was taken from the growth present in the oral cavity. Impression smears were prepared from various organs showing lesions. The cytology smears were fixed in methanol, air dried and stained with Giemsa, Leishman and combination of Leishman-Giemsa. The oral growth and organs showing lesions were collected and fixed in 10 percent neutral buffered formalin for histopathological examination. The tissue sections were cut into 4 µm thickness and subjected to routine H&E staining.

On gross examination, multiple blackish nodular growths were seen in the left lateral aspect of the oral cavity, upper and lower gums as well as in oral commissures (Fig. 1). There were stray tiny greyish black nodules scattered over the lung parenchyma. Spleen showed two large circumscribed black coloured elevated nodules on lateral borders with few raised areas on the dorsal surface (Fig. 2). There were no observable gross lesions in the heart and

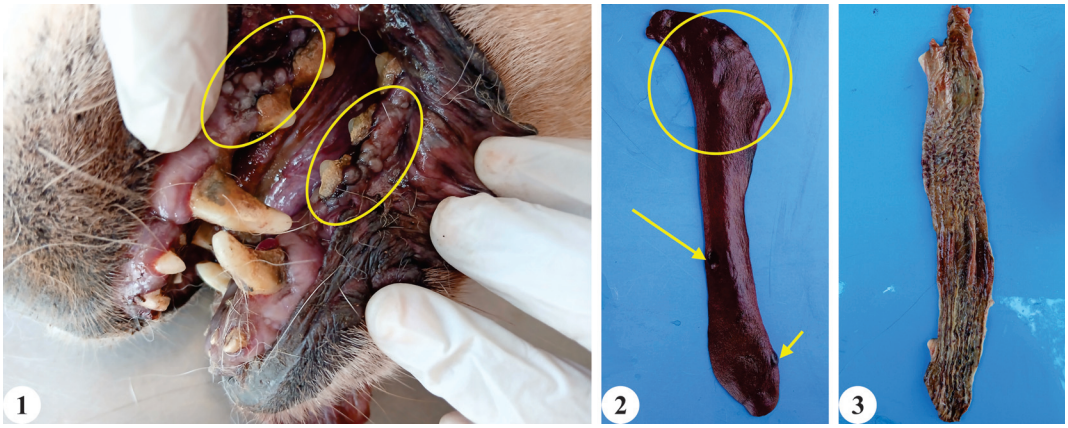

Fig. 1. Multiple black coloured nodular growth on the left lateral aspect of the oral cavity (circles); **Fig. 2.** Circumscribed blackish nodules (arrows) with multiple raised areas (circle) noticed on the splenic parenchyma; **Fig. 3.** Large intestine showing thickened mucosa with black discolouration and haemorrhage.

liver except for mild hepatic congestion and subcapsular haemorrhage in liver.

The gastric mucosa was thickened with few scattered ulcers. Intestinal serosa revealed stray blackish areas and the mucosa exhibited severe thickening with haemorrhage and linear to nodular blackish areas (Fig. 3). Both the kidneys were shrunken and loss of renal architecture with cystic, pitted and uneven surface (Fig. 4); while on cut section, there was focal blackish areas in corticomedullary junction. The mucosa of urinary bladder and prostate gland were severely thickened.

FNAC from the oral growth revealed clusters of melanin-laden melanocytes with anisocytosis, anisokaryosis, prominent and multiple nucleoli, intracytoplasmic coarse granular black coloured melanin pigments (Fig. 5). In few cells, melanin granules totally masked the nucleus and appeared as dark areas. Similarly, the impression smears from the black nodular areas of the spleen and lungs also exhibited scattered cell population of melanin-laden neoplastic melanocytes.

Histopathological examination of the mass from the oral cavity exhibited replacement of various layers of normal stratified squamous epithelium with numerous melanin-laden melanocytes (Fig. 6). The lung section showed metastatic blackish melanotic nodule in the peribronchiolar areas (Fig. 7). Liver exhibited severe necrosis with occasional melanin-laden melanocytes in the sinusoidal space. There were huge population of melanin-laden melanocytes replaced the normal lymphatic tissue and lymphoid cells of spleen (Fig. 8). Masson Fontana stain was employed on the sections of spleen to differentiate melanin and haemosider in pigment, where those were found to be positive. The intestine showed severe destruction of intestinal villi and crypts of lieberkuhn with severe submucosal congestion and focal infiltration of melanin-laden melanocytes. There were stray melanin-laden melanocytes in the renal tubular basement membrane and severe fibrous tissue proliferation with mononuclear cell infiltration noticed in kidney sections (Fig. 9). Prostate revealed fibrous tissue proliferation with atrophied glandular tissue.

Most of the malignant melanoma occurs either as sessile mass or pedunculated growth with small stalk. The aggressiveness is low in the case of pedunculated mass when compared to sessile growth[3]. The exact mechanism is not clear; this is mainly due to biological nature of neoplasm. In the present case, there was multiple blackish sessile nodular growth noticed in the oral cavity with multicentric metastasis. The gross morphology and metastatic potential of the blackish sessile nodular growth in oral cavity is very well correlated with the earlier report[3].

The clusters of melanin-laden melanocytes with prominent multiple nucleoli and intracytoplasmic coarse granular blackish pigments were noticed in the FNAC samples of oral growth. Similar cytological features were also observed in spleen and lungs. These cytological observations were in accordance with the findings of earlier authors[13]. Additionally, the cytological findings were well illustrated by the histopathological features of the oral growth and other affected organs.

In the present case, the primary tumour was noticed in the oral cavity and metastasis to various organs like of spleen, lung, kidney and intestine which was evidenced in both gross and microscopic lesions. The multicentric melanoma noticed in the present case might be due to metastasis via the lymphatic system, regional lymph nodes and subsequent spread to various organs[3,14].

Multiple tiny greyish/blackish nodules in lung and spleen noticed in the present case, might be due to metastasis of neoplastic melanocytes from the primary site of oral melanoma[15]. Shrunken kidneys and stray focal blackish area noticed in the corticomedullary

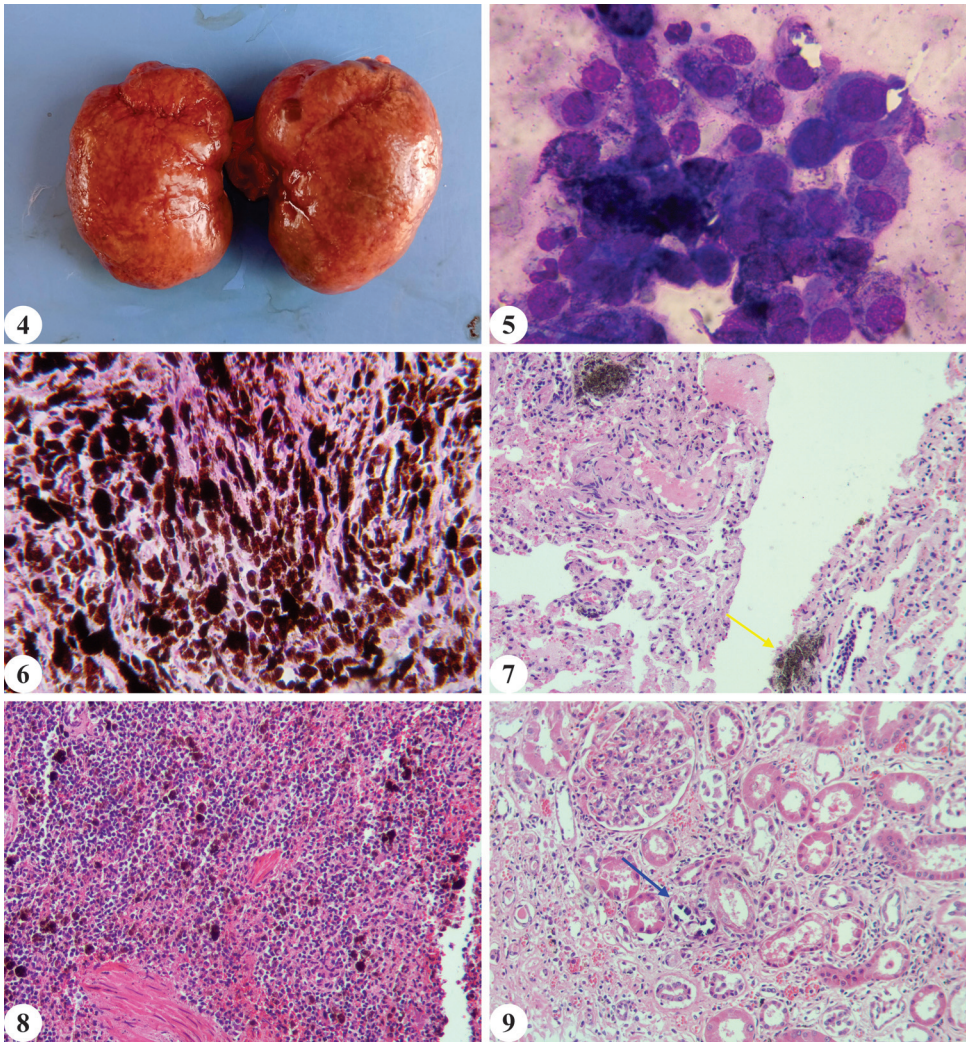

Fig. 4. Shrunken kidneys with pitted, cystic and granular surface; **Fig. 5.** Clusters of cells showing anisocytosis, anisokaryosis and prominent nucleoli with intra cytoplasmic blackish melanin pigment (Giemsa x400); **Fig. 6.** Oral cavity growth showing neoplastic melanocytes replacing the normal oral stratified squamous epithelium (H&E x400); **Fig. 7.** Lung revealing metastatic blackish melanotic nodule (arrow) in peribronchiolar areas (H&E x100); **Fig. 8.** Spleen revealing melanin laden neoplastic melanocytes in the splenic parenchyma (H&E x100); **Fig. 9.** Kidney section showing melanin laden melanocytes in the tubular basement membrane (arrow) with fibrous tissue proliferation and mononuclear cell infiltration (H&E x100).

junction might be due to the chronic inflammatory response related with neoplastic conditions and senility. It was evidenced in histopathology by fibrous tissue proliferation in interstitium and presence of melanin laden melanocytes in the tubular basement membrane. The prostate gland exhibited mucosal thickening and atrophy of glandular tissue which might be due to advanced age.

A notable feature observed in this study was gross blackish thickening of the intestinal mucosa and presence of melanin laden melanocytes within the submucosa in microscopy though intestinal malignant metastatic melanomas are uncommon in dogs[16].

Metastatic malignant oral melanoma leading to aggressive local invasiveness and widespread metastasis to various vital organs resulted in organ failure and fatal death.

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Conflicts of Interest: None

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SHORT COMMUNICATION

Concurrent infection of histomoniasis and colibacillosis in ducklings

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ABSTRACT

Ducklings (aged 45-50 days) from a nomadic flock had mortalities of 4-5 birds per day since one week was reported. Systematic postmortem was conducted. Samples were collected for histopathological, microbiological and parasitological studies. Gross examination revealed thin whitish membranous deposits over the pericardium and liver. Pericardium was tightly adhered with the heart. Caecum was slightly distended and showed multifocal, firm creamy yellowish-white adherent foci over the mucosa. Microscopically, chronic fibrino-purulent pericarditis, epicarditis, fibrinous perihepatitis, diptheritic typhlitis with trophozoites of *Histomonas meleagridis* was observed. *Histomonas meleagridis* was confirmed by parasitological examination. *Escherichia coli* was isolated from heart blood and liver swabs. Histomonads exacerbate the illness when concurrent infection with pathogenic bacteria prevails. The present study described the co-infection of histomoniasis and colibacillosis in ducklings under natural condition.

Keywords: Chronic granulomatous pericarditis, diphtheritic typhlitis, duckling, *Escherichia coli*, *Histomonas meleagridis*

Histomoniasis is a protozoan disease of gallinaceous birds caused by unicellular parasite *Histomonas meleagridis* belonging to the phylum Parabasalia, class Tritrichomonadea, order Tritrichomonadida and family Dientamoebidae[1]. Turkeys are highly susceptible with acute mortality reaching up to 100%[2], whereas Ring-necked pheasants are found to be relatively resistant[3]. However, histomoniasis found to occur in domestic and wild gallinaceous birds[2,4,5], it is rare in water fowls especially in ducks[6,7]. Histomoniasis is also reported to cause mortality in established infection of *H. meleagridis* along as a co-infection with internal pathogenic bacteria and intestinal nematodes *(Ascardia galli* and *Heterakis gallinarum)*[8]. Concurrent infection with grave malady of histomoniasis is reported on pair with *Enterococcus fecalis* in ducks[9] and with *Eimeria tenella*[10], colibacillosis[4] and ascariasis[11] in chickens. Histomoniasis was reported to occur in the absence of possible vectors by cloacal route[12]. The present report documented the co-infection of histomoniasis and chronic colibacillosis as a natural infection in an Indian runner duck.

A nomadic duck farmer reported a mortality of four to five ducklings per day since a week in 42 to 50 days old age groups in a flock holding 4000 birds. History showed that ducklings had yellowish watery diarrhea, twisting of head and limping for two to three days before death. Three dead ducklings were brought to the Department of Veterinary Pathology, Veterinary College and Research Institute, Orathanadu, Thanajvur, Tamil Nadu for postmortem examination. Complete and systematic postmortem examination was carried out. Representative tissue samples from coelomic organs and brain were collected in 10% formalin for histopathology. Heart blood and liver swabs were collected in sterile tubes for microbiological study. Caecal luminal contents were collected in sterile container for parasitological study.

Tissue samples were processed as per standard paraffin embedding technique. Tissue sections of 4-5 µm thickness were prepared and stained with haematoxylin and eosin staining protocol[13]. Duplicate sections were stained with MSB technique to demonstrate fibrin[14]. Heart blood and liver swabs were processed as per standard bacteriological procedure. Heart blood swab was nourished in nutrient broth and further streaked in MacConkey agar and Eosin Methylene Blue (EMB) agar. Caecal contents were subjected to direct wet film examination following centrifugation under light microscope.

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Grossly, carcasses were thin and feathers around the vent were matted with creamy whitish pasty contents. Birds had sunken eyes (Figs. 1 & 2). On pressing the lower abdomen near cloaca, whey coloured (yellowish green tint) watery discharge was found (Fig. 3). Internally, the liver was covered with thin, whitish pseudo-membranous layer (Fig. 4). The heart revealed thick, chalky-white pericardium. Pericardium was tightly adhered

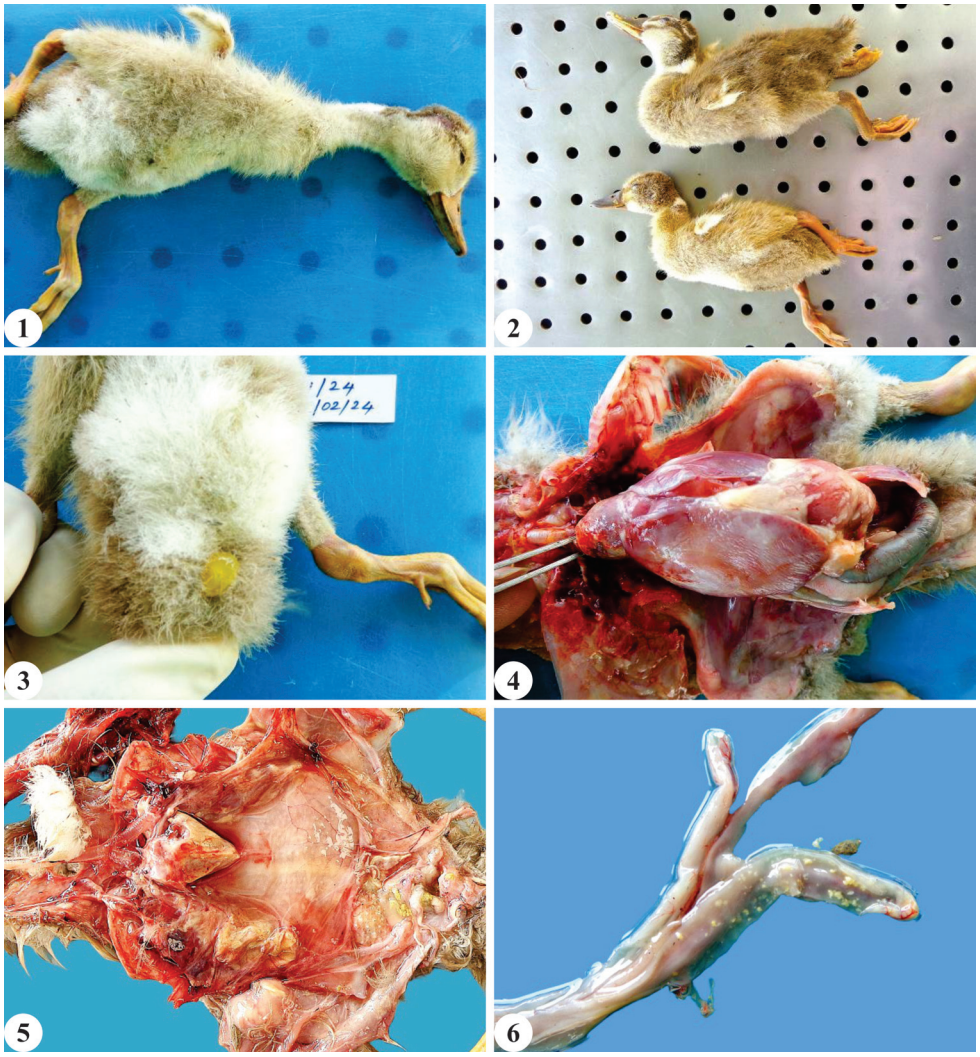

Fig. 1. Duckling with ruffled down feathers; **Fig. 2.** Ducklings with torticollis; **Fig. 3.** Yellowish-green (Whey-like) cloacal discharge; **Fig. 4.** Thin, whitish membranous deposits over liver; **Fig. 5.** Yellowish white, thick adherent pericardium; **Fig. 6.** Multifocal, creamy yellowish-white firm diphtheritic areas in the mucosa of caecum.

with the epicardium (Fig. 5). Both the caecum was slightly distended and contained yellowish watery contents. Mucosa of caecum revealed multifocal, creamy yellowish white, raised, firm diphtheritic foci which were found adhered to the underlying tissue (Fig. 6). Other visceral organs showed mild to moderate congestion. Brain revealed mild congestion of meninges.

Microscopically, pericardium was thick and revealed fibrinopurulent inflammation characterized by deposition of fibrin with necrosis and degenerative heterophils, bacterial clumps surrounded by mononuclear cells and row of multinucleated giant cells (MGCs) (Fig. 7). MGCs' were foreign body type. MGCs' showed mixed population of Langhan's type with peripheral row of nuclear arrangement (Fig. 8) and as foreign body type with central nuclear cluster as well. Underlying epicardium revealed superficial infiltration with mononuclear cells into the myocardial fibers. In another carcass, pericardial cavity was filled with fibrino-purulent necrotic and highly cellular contents with bacterial colonies. Pericardium was found to be strongly adhered with the underlying epicardium which was anchored by columnar type giant cells. These giant cells were taller and had two rows of multiple nucleus (4-5) arranged one above the other pointing on either side; one from the visceral pericardium and another from the epicardium (Fig. 9). Underlying epicardium showed inflammatory cell infiltrations. Liver revealed thickening of Glisson's capsule with inflammatory cell infiltrations (Fig. 10). Parenchyma showed moderate to severe congestion, atrophy of hepatic cords and vacuolar degeneration of hepatocytes. Caecum revealed multifocal diphtheritic typhlitis characterized by mucosal necrotic plug adhered with the underlying mucosal cells (Fig. 11). Mucosal epithelium and glands showed atrophy with intraluminal trophozoites of *Histomonas meleagridis* (Fig.

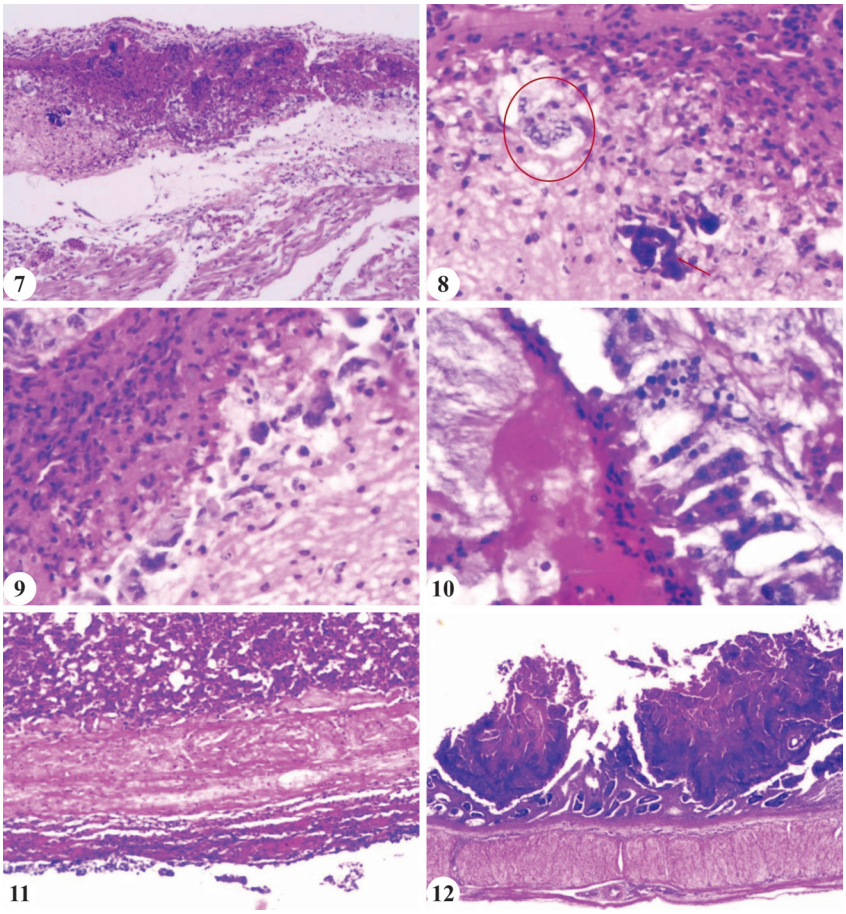

Fig. 7. Fibrino-purulent exudate with chronic pericarditis, MNC infiltrations into the epi and myocardium (H&E stain x100); **Fig. 8.** Chronic pericarditis, Giant cells (circle) and bacterial colonies (arrow) (H&E stain x400); **Fig. 9.** Degenerated heterophils and row of Langhans type giant cells (H&E stain x400); **Fig. 10.** Columnar type giant cell strands showing row of multinucleation of anchoring the epicardium and visceral pericardium (H&E stain x400); **Fig. 11.** Perihepatitis showing thickening of Glisson's capsule and peripheral MNC infiltration and inflammatory exudate (H&E x100); **Fig. 12.** Diphtheritic necrotic plaques adhered with mucosa (H&E stain x100).

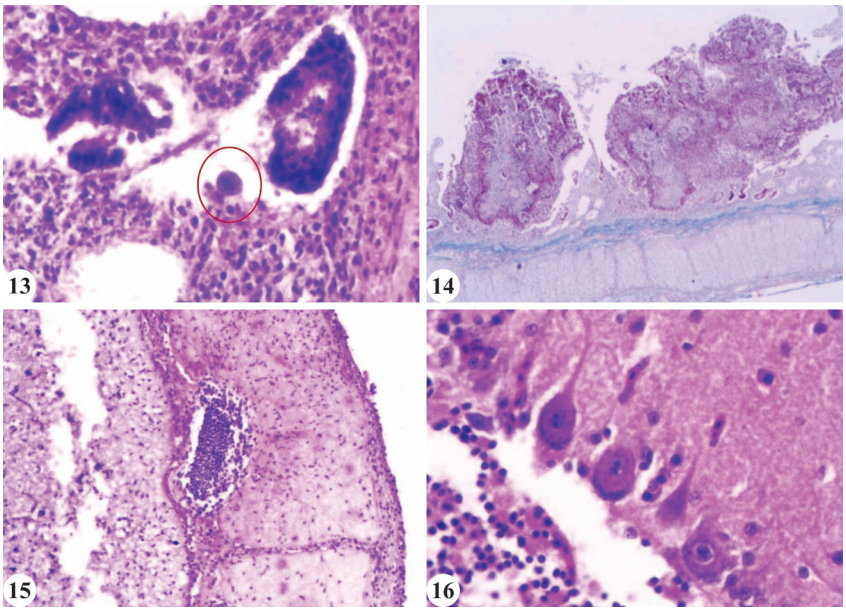

Fig. 13. Trophozoites of *Histomonas Meleagridis* (circle) with in the mucosa of caecum (H&E stain x400); **Fig. 14.** Red coloured fibrin in the diphtheritic plaques (MSB technique x100); **Fig. 15.** Sub-acute eosinophilic leptomeningitis (H&E x100); **Fig. 16.** Peripehral condensation of Nissl substance in Purkinje cells of cerebellum (H&E stain x400).

12). Necrotic plug showed high density of fibrin with MSB staining technique (Fig. 13). Meninges revealed subacute eosinophilic leptomeningitis (Fig. 14). Cerebellum showed mild to moderate peripheral condensation of Nissl substances in Purkinje cells (Fig. 15).

Caecal contents on wet film examination revealed numerous motile single flagellate trophozoites suggestive to *Histomonas meleagridis*. Dried smear stained with H&E stain revealed oval to round bodies surrounded by clear space (Fig. 16). *Escherichia coli* was isolated from heart blood and liver swabs. Colonies from heart blood swab produced green metallic sheen in EMB agar and produced typically bright pink, smooth, circular, moist colonies in MacConkey agar.

Histomoniasis was known to cause entero-hepatitis characterized by necrotic hepatitis and diphtheritic typhlitis. Turkeys were long known to be the susceptible host for *H. meleagridis*[15]. It was documented that other gallinaceous birds such as chicken, Pheasants, Partridges, guinea fowl, geese, ducks and peafowl were found to suffer with *H. meleagridis* infection both naturally and experimentally[3-5,7,11,16-18]. Histomoniasis was found to be rare in water fowls[8]. Ducks were reported to be an unsatisfactory host for establishment of *H. meleagridis* infection[6]. Experimental studies on histomoniasis in mule and Muscovy ducks do not exhibit any overt clinical signs other than diarrhea when compared to turkeys[7]. In parallel, Abd El-Wahab *et al.*[16] reported that fattening turkeys affected with histomoniasis had typical lesions in liver but did not show any apparent clinical signs of the disease before slaughter. The above documentary evidence, although puzzling, natural infection with *H. meleagridis* resulted in yellowish (whey-like) watery diarrhea in ducks as observed in the present study.

No apparent gross lesions of histomoniasis were observed in liver except mild caecal lesions in the present study and was in accordance with earlier reports[6,7]. Callait-Cardinal *et al.*[7] documented that histomoniasis affected ducks showed thickening of caecal wall with partial luminal occlusion with caecal core. The findings of multifocal, bran-like necrotic areas in the caecal mucosa was in parallel with the findings of Lund *et al.*, (1974)[6] and de Araujo *et al.*, (2015)[19], who reported that the infection with *H. meleagridis* revealed rudiment of adherent core in the caecal mucosa of experimentally infected geese and yellowish friable caseous contents over the mucosa in natural infection of free-range chicken respectively. On whole, *H. meleagridis* seldom associated with overt necrosis of liver and caecum put forth by Clarke *et al.*, (2017)[4] was found to be justifiable in the present report with lack of discrete lesions of histomoniasis.

Histologically, multifocal diphtheritic necrotic plaques with the presence of trophozoites of *H. meleagridis* recorded in the present study was in line with earlier report in duck[7] and in chicken[19]. Earlier, necro-fibrinous typhlitis was recorded in mule and Muscovy ducks experimentally infected with *H. meleagridis*[7]. It was suggested that proteases released by the protozoa likely to cause digestion and destruction of epithelial cells of the caecal mucosa which subsequently leads to necrotic typhlitis and formation of caecal core[1]. Subacute eosinophilic meningitis observed in the present study might possibly be responsible for nervous signs exhibited by ducks before death. Although histomonads were not found in the meningeal lesion of the present study, molecular localization of histomonads was reported in multisystemic infection of histomoniasis affecting visceral organs including brain[20].

It was postulated that *H. meleagridis* alone does not represent substantial danger to duck[7] rather, co-infection with *Eimeria tenella*, pathogenic bacterium such as *Escherichia coli*, *Bacillus subtilis* or *Clostridium perfringens* and round worm, *Ascaridia galli* could aggravate the clinical signs, exuberate the illness and increase the mortality rate[10,16,11,21]. It was documented that haemorrhagic enteritis virus occurred currently with histomoniasis in turkeys[18]. Further it was reported that colibacillosis was much frequent co-infection following histomonas infection representing that *E. coli* translocates from the gut to internal organs via blood stream probably due to loss of gut membrane integrity[2,22]. The above statement was acceptable in the present study where affected ducklings showed fibrous perihepatitis and granulomatous pericarditis and epicarditis characteristic of colibacillosis. Further the *E. coli* infection in the present malady was confirmed by microbiologial study. Histological changes in pericardium and epicardium suggests that the malady induced by bacteria was of chronic lesions.

Histomoniasis was once thought to be primarily transmitted by ingestion of embryonated eggs of *Heterakis gallinarum* from neighbouring chicken flock to turkeys. Incidence of histomoniasis in chicken and other gallinaceous birds in the absence of caecal worm and vector/reservoir host such as darkling beetles, earthworm and lesser mealworm suggested the possibility of direct spread via cloacal route of infection[12,23]. This was justified with the primary lesion of histomonaisis in bursa of Fabricius of desi chicken infected with *H. meleagridis* with mild caecal lesion[21,24]. Cloacal drinking/cloacal kissing was proposed to be one of the reasons of direct spread for *H. meleagridis*[1,24]. High moisture droppings, huddling of ducks in closed rearing in backyard farming pose risk for cloacal kissing in ducks[12]. The above statement was plausible in the present study where no vectors (caecal worm or eggs) were found by parasitological examination.

CONCLUSION

The present study concludes that mortality of ducks were due to co-infection with *H. meleagridis* and *E coli*. Direct spread of histomonas followed by secondary *E.coli* infection aggravated the disease. Further, the chronic colibacillosis with newer pericardial lesions thought to be rare findings in ducks. On par with other gallinaceous birds, ducks were found to be susceptible for natural infection by *H. meleagridis* followed by secondary colibacillosis. Additionally, *Histomonas* established in caecum with tone-down lesions in the present study suggested that ducks may act as reservoir and or carrier host for *H. meleagridis* to other poultry when reared together. Similar statement as already proposed by earlier workers[7] also need to be investigated.

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SHORT COMMUNICATION

Pathomorphological studies on respiratory Aspergillosis in a japanese quail farm

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ABSTRACT

Three ailing and eight breeder quail carcasses of both sexes at 10 weeks old were brought to Department of Veterinary Pathology for clinical diagnosis and postmortem examination respectively with the history of regular mortality for the past five days. Clinically, ailing birds showed dullness, depression with respiratory distress and nasal discharges. Gross examination revealed yellow to whitish caseous nodules on the lung parenchyma. Air sacs were thickened with multiple tiny whitish nodules. Histopathological examination of air sacs revealed granulomatous inflammation characterised by the presence of tubuliform structures which were surrounded by caseous exudate and infiltration of macrophages and lymphocytes. Lungs revealed caseous central core mass contained numerous septate fungi and was surrounded by heavy infiltration of heterophils, macrophages, lymphocytes and giant cells. This was confirmed by special stains namely Periodic acid-Schiff (PAS) and Grocott methenamine silver nitrate (GMS) which showed pink and black coloured septate hyphae respectively. Based on the cultural characteristics, gross, cytology, histopathological and histochemical findings, it was concluded as aspergillosis infection in the breeder quail farm.

Keywords: Air sacs, Aspergillosis, caseous exudate, giant cells, lungs, quail

Aspergillosis is a non-contagious fungal disease that can affect humans, animals and birds due to inhalation of fungal spores found in feed, bedding and litter materials. It is a ubiquitous conidial saprophytic fungus that affects almost all variety of birds like chicken, ducks, quails and wild birds and often leading to high morbidity and mortality rates[1,2]. Avian aspergillosis is caused by different species: *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus* and *Aspergillus glaucus*. Among these, *Aspergillus fumigatus* is the most common pathogen involved in the avian aspergillosis[3].

The primary route of transmission for aspergillosis is through inhalation and it affects lower respiratory disease mainly air sacs and lungs[2,4]. The fungal hyphae on air sacs and lungs either produce localized acute infection like air sacculitis and bronchopneumonia or chronic granulomatous inflammation. In some cases, hematogenous spread causes chronic granulomatous lesions in all visceral organs[5]. However ocular and neural form of aspergillosis also reported in birds[2]. In some cases, it may cross Blood Brain Barrier (BBB) and localise in brain tissue causing encephalitis marked by neurological symptoms[6]. The present report describes the pathomorphological studies of respiratory aspergillosis in breeder Japanese quails.

A breeder Japanese quail flock with the capacity of 350 birds had daily mortality of seven to eight birds regularly. Three ailing (One male and two female) birds and eight (three female and five male) dead Japanese breeder quail carcasses of both sexes were presented to the Department of Veterinary Pathology, Veterinary College and Research Institute, Namakkal for disease investigation and postmortem examination respectively. Detailed clinical examination was done and clinical signs were noted. Systematic necropsy was carried out and gross lesions were recorded.

Impression smears taken from the cut section of nodules from lungs and air sacs were subjected to Gram staining, acid fast staining and lactophenol cotton blue staining to rule out the etiological agent. Lung and air sac tissue suspensions were inoculated into Sabouraud dextrose agar (SDA) and incubated at 37^{O}C for one week[7]. Smears were prepared from the cultured fungus and subjected to lactophenol cotton blue staining technique for identification of fungal organisms.

Organs showing lesions were collected in 10 percent neutral buffered formalin. Paraffin embedded tissue sections were cut at four-micron thickness and stained with Haematoxylin and Eosin (H&E). Additionally, histochemistry techniques like PAS and GMS were performed for further confirmation[8].

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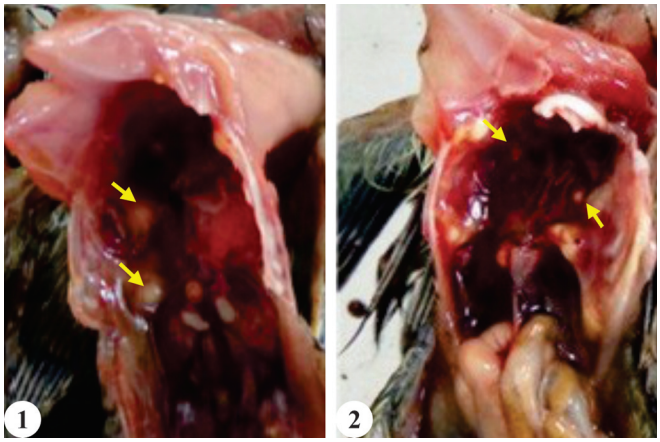

Fig. 1. Air sacs showing whitish nodules; **Fig. 2.** Lungs revealing yellowish white nodules studded in the parenchyma.

Mortality was noticed within 24-48 hrs of onset of symptoms about 37 birds out of 350 breeder quails over a period of five days. Clinically, ailing birds were emaciated, anorectic, dull, depressed and showed respiratory distress, nasal discharge and incoordination. On necropsy, external examination revealed emaciated and dehydrated carcasses with ruffled feathers. The keel bone was prominent with atrophy of breast muscles. Internal examination revealed thickening of both thoracic and abdominal air sacs with scattered whitish nodules (Fig. 1). The cut section of nodules exhibited cheesy mass of varying diameter (2-5 mm). There were yellowish white caseous nodules studded on the lung parenchyma (Fig. 2). However, the other viscera were normal devoid of any specific lesions.

In cytology, impression smears from crushed nodules of lung and air sacs showed no observable bacteria or acid-fast organisms in Gram staining and Acid-fast staining respectively. Whereas, numerous branched septate fungal hyphae along with characteristic conidial structures were noticed in Lactophenol Cotton Blue staining under light microscopy. *Aspergillus fumigatus* was recovered from SDA culture of lung and air sacs. Dark green velvety colonies appeared within 48 hrs with white reverse pigmentation. The colony showed spiny green conidiophores with the short and smooth spores on the upper half of the vesicle.

Histologically, air sacs showed nodule with caseous necrotic area in the centre surrounded by fibrous capsule (Fig. 3). It showed tubuliform fungal hyphae in the centre surrounded by heavy infiltration of heterophils, lymphocytes and macrophages (Fig. 4). Lungs showed multifocal caseous necrotic areas surrounded by heterophils, lymphocytes, macrophages and numerous giant cells (Fig. 5). There were pink coloured septate and branching hyphae observed in PAS-stained lung sections (Fig. 6). In GMS staining, black coloured fungal hyphae were dispersed in the caseous necrotic mass as well as in the surrounding pulmonary parenchyma (Fig. 7).

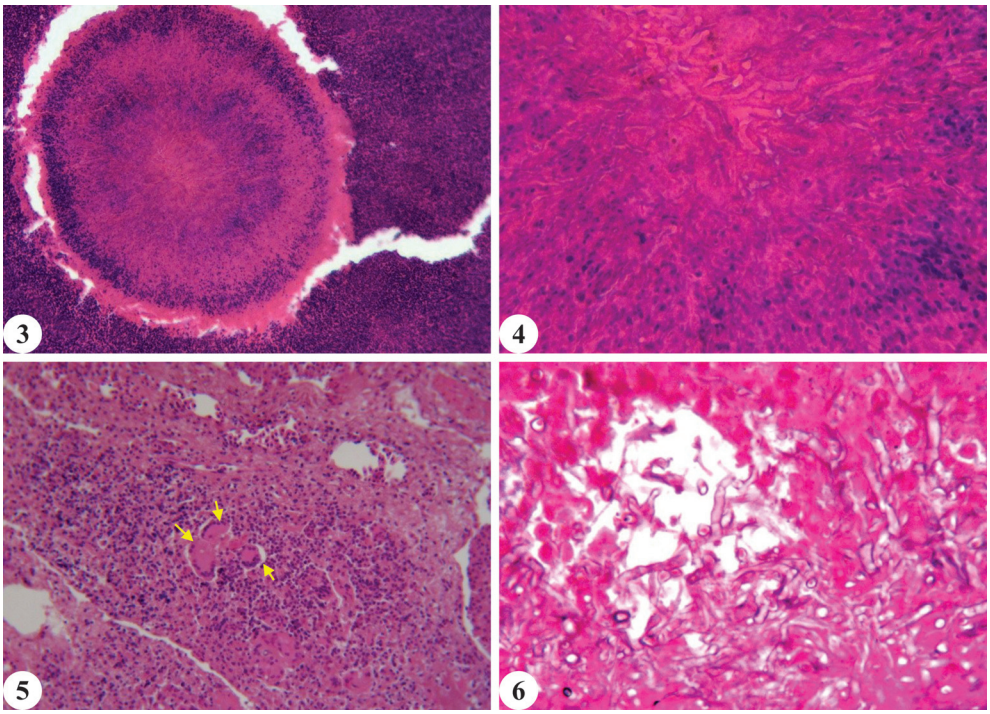

Fig. 3. Air sac showing caseous mass containing fungal hyphae surrounded by inflammatory cells and fibrous capsule (H&E x100); **Fig. 4.** Air sac showing caseous necrosis with radiating fungal hyphae and infiltration of inflammatory cells (H&E x400); **Fig. 5.** Lung showing granulomatous reaction with heterophils, lymphocytes and giant cells (H&E x100); **Fig. 6.** Lung showing caseous mass containing numerous pink coloured fungal hyphae (PAS x400).

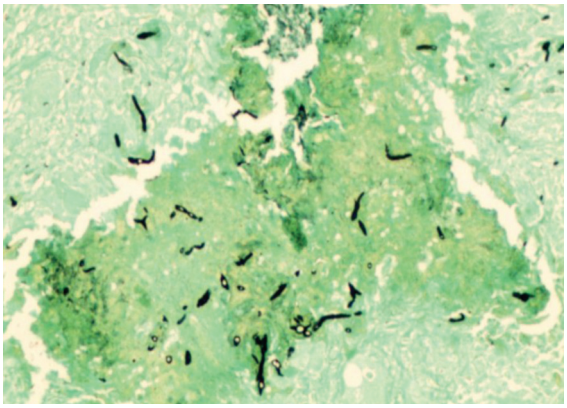

Fig. 7. Lung showing blackish fungal material in caseous mass and surrounding parenchyma (GMS x400).

Based on the colonial morphology, reverse pigmentation and cytology by using LCB technique, the isolated fungus was identified as *A. fumigatus*. In addition, histomorphology and histochemistry further confirmed the fungal pneumonia by *A. fumigatus.*

The affected birds exhibited the symptoms of acute aspergillosis *viz.* anorexia, respiratory distress, depression and death and these clinical signs were supported by the earlier report [9]. In the present study, lesions were confined to the respiratory system rather than a systemic manifestation[10]. The extremely small size of *A. fumigatus* conidia (2-3 μm) enables them to evade the mucociliary clearance mechanism and allow the conidia to reach the lower respiratory tract, facilitating pulmonary deposition and potential colonization[10].

The air sacs appeared abnormally thickened and opaque due to caseous exudates which was deviated from their thin and transparent appearance. Similar air sac lesions were also recorded by previous author[11]. Granulomatous lesions in lungs and air sacs are the hallmark of pulmonary aspergillosis. Microscopic lesions showed typical granulomatous lesion in lungs and air sacs with central caseous necrotic mass with fungal hyphae surrounded by fibrous capsule and infiltration of lymphocytes and giant cells. These histopathologic observations were very well correlated with gross pathology and were in accordance with recent reports on pulmonary aspergillosis[1,12].

Although Japanese quails are generally hardy, fungal pneumonia caused by aspergillosis may occur due to predisposing immunosuppressive conditions such as infectious bursal disease and chicken infectious anemia. These diseases are more commonly found in nearby commercial layer farms, can increase the susceptibility of quails to opportunistic fungal infections.

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SHORT COMMUNICATION

Pathology of infectious canine hepatitis in captive Indian wolves (*Canis lupus pallipes*): An outbreak study

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ABSTRACT

This study reports an outbreak of infectious canine hepatitis (ICH) in a captive pack of four sub-adult Indian wolves *(Canis lupus pallipes)*. The first wolf showed signs of lethargy, loss of appetite and fever before dying suddenly. Despite symptomatic treatment, the remaining three wolves also died within 24 hours, showing similar symptoms. Necropsy examination of all wolves revealed a soft, enlarged and discoloured liver with widespread haemorrhages, which were also present in other internal organs. Histopathology of the liver showed severely engorged and dilated vasculature and sinusoids, areas of haemorrhages, degeneration and necrosis of hepatocytes and basophilic intranuclear inclusion bodies in hepatocytes and Kupffer cells. Similarly, vascular changes were also seen in the lungs, kidneys, spleen, heart and intestines. Immunohistochemistry confirmed Canine Adenovirus-1 (CAdV-1) antigen in hepatocytes, endothelium and Kupffer cells, while PCR detected CAdV-1 in liver. This study highlights the significance of ICH in Indian wolves and its implications for their conservation.

Keywords: Canine Adenovirus-1, indian wolf, liver, pathology, PCR

The Indian wolf *(Canis lupus pallipes)*, classified as endangered and listed under Schedule I of the Wildlife Protection Act (1972) in India, faces serious conservation threats due to habitat loss, fragmentation and emerging infectious diseases[1,2]. Canine adenoviruses, CAdV-1 and CAdV-2, belong to the family *Adenoviridae* and genus *Mastadenovirus*[3]. CAdV-1 is the causative agent of infectious canine hepatitis (ICH), a highly contagious disease affecting primarily canids, as well as ursids and mustelids worldwide[4,5]. CAdV-1 mainly targets hepatocytes and endothelial cells, causing systemic illness and widespread tissue damage[5,6]. In contrast, CAdV-2 primarily infects the respiratory tract, leading to infectious canine tracheobronchitis (kennel cough)[4]. CAdV-1 is environmentally stable, capable of surviving for extended periods and spreads through direct contact, contaminated fomites or urine from infected animals[7]. While vaccination with modified live CAdV-2 vaccines provides cross-protection and has reduced the prevalence of CAdV-1 in domestic dogs in developed regions[6], unvaccinated dogs and wildlife reservoirs such as foxes, wolves and bears continue to support viral circulation and pose a risk of re-emergence[7]. In India, ICH has been reported in unvaccinated domestic dogs and occasionally in wild canids, including dholes (Asiatic wild dogs) and foxes[8]. The present study documents an outbreak of ICH in captive Indian wolves, highlighting the ongoing threat posed by CAdV-1 to wildlife conservation.

A sudden death in a captive pack of four sub adult Indian wolves two male and female each occurred at the Nawab Wajid Ali Shah Zoological Garden, Lucknow, Uttar Pradesh. Initially, one wolf showed signs of anorexia, lethargy, high fever (104.2^{O}F) and bloody diarrhoea, leading to sudden death (Fig. 1a). The remaining three wolves died within 24 hours, despite receiving symptomatic treatment. Blood and serum samples were collected prior to death for laboratory analysis. A systematic necropsy was performed and tissue samples from major organs were collected in 10% neutral-buffered formalin and on ice. Formalin-fixed tissues were processed and stained with Haematoxylin and Eosin for histopathological evaluation. Indirect immunohistochemistry (IHC) was conducted on liver sections using mouse anti-CAdV-1 monoclonal antibody (VMRD, USA)[9]. DNA was extracted from liver tissues and PCR targeting the E3 gene was performed following established protocols[10].

Serum biochemical analysis revealed elevated liver enzymes (ALT: 418±52 IU/L; ALP: 332±34 IU/L) indicating hepatic damage. Major gross lesions included congested or icteric conjunctival

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Fig. 1. Pathological findings of ICH in Indian wolves. **a.** Clinically affected wolf showing dull and recumbent in position. **b.** Weak and dehydrated carcass of wolf died due to ICH. **c.** Congested discoloured and enlarged liver with widespread haemorrhages and distended gall bladder. **d.** Bloody contents in the lumen of small intestine due to ICH.

membranes, dehydrated and weak carcasses, an enlarged, soft, severely congested, discoloured and haemorrhagic liver with rounded borders and distended gall bladder (Fig. 1b & c). Haemorrhages were also seen throughout the gastrointestinal tract, which contained bloody contents (Fig. 1d). The congestion and haemorrhages were also observed in lungs, spleen, heart and kidneys. Histopathological examination of the liver showed diffuse engorgement and dilatation of vasculature including sinusoids, areas of haemorrhages, hepatocytic degeneration and necrosis, increased Kupffer cell activity and basophilic intranuclear inclusion bodies in hepatocytes, Kupffer cells and endothelial cells (Fig. 2b & c). Severe vascular lesions like highly engorged blood

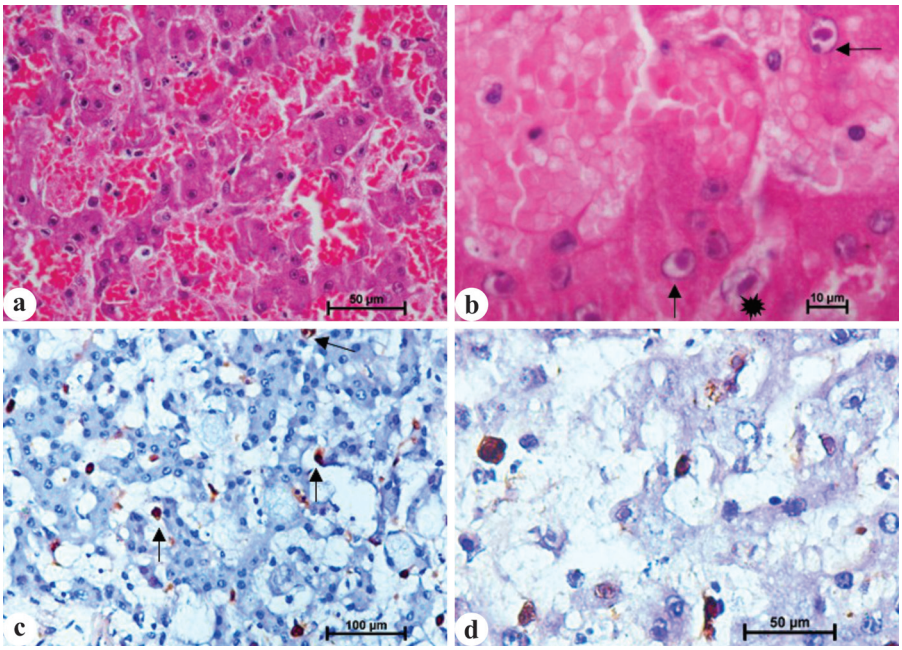

Fig. 2. Microscopic findings of ICH in Indian wolves. **a.** The liver section showing severely dilated and engorged sinusoids with degeneration and necrosis of hepatocytes (H&E x400). **b.** Basophilic intranuclear inclusion bodies in the hepatocytes (arrow) and endothelial cells (star) (H&E x1000). **c & d.** Liver section showing positive immune staining in hepatocytes, endothelial cells and Kupffer cells to CAdV-1 antigen (arrow). (IHC DAB x200, x400).

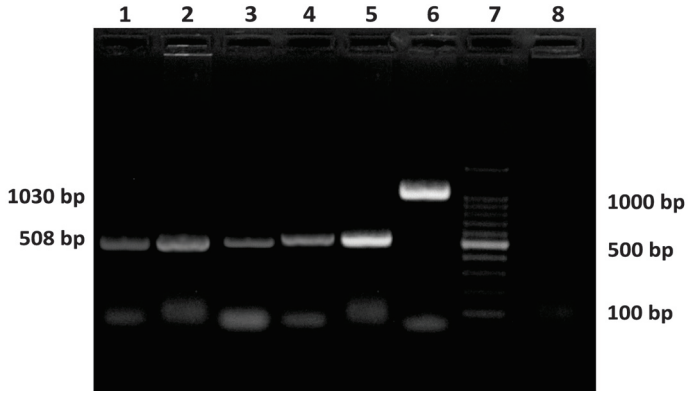

Fig. 3. Detection of Canine adenovirus virus in Indian wolves by conventional PCR assay. Lane 1-4: Liver samples positive for CAdV-1 (508 bp), Lane 5: Positive control CAdV-1 (508 bp), Lane 6: Positive control CAdV-2 (1030 bp), Lane 7: 100 bp ladder, Lane 8: Negative control.

vessels with haemorrhages were also observed in the lungs, kidneys, spleen, intestine and heart. Immunohistochemistry showed positive labelling for CAdV-1 antigen in hepatocytes, Kupffer cells as well as endothelium (Fig. 2d & e). PCR confirmed the presence of CAdV-1, yielding a 508 bp product specific to the E3 gene (Fig. 3).

Necro-haemorrhagic hepatitis accompanied by hepatomegaly is commonly reported in CAdV-1 infection among both domestic and wild canids, which is consistent with the findings of this study[5,6]. CAdV-1 infection in foxes has occasionally been referred to as 'fox encephalitis' due to the presence of associated neurological signs[11]. However, no neurological symptoms were observed in the present outbreak. In India, data on the prevalence of CAdV-1 in wild canids remain limited, yet the virus poses a significant threat to species like the Indian wolf, which shares close genetic ties with domestic dogs[8]. These wolves often inhabit areas that overlap with domestic dogs, jackals *(Canis aureus)* and other wildlife, increasing the risk of interspecies transmission[7]. The absence of widespread vaccination in rural areas, combined with overlapping territories of domestic and wild canids, may facilitate the continued circulation of CAdV-1[6,7]. The detection of CAdV-1 in captive Indian wolves underscores its potential threat to other vulnerable wildlife species. Its presence in a zoological setting raises concerns about spillover to susceptible animals such as foxes, jackals and bears. These findings highlight the urgent need for regular health monitoring, active surveillance and vaccination of captive wild canids to prevent and control the spread of CAdV-1.

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CASE REPORT

Concurrent Traumatic Reticulo Pericarditis (TRP) with Theileriosis in HF-Cow

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ABSTRACT

A 4-year-old Holstein Friesian cow was presented to the Department of Veterinary Pathology, GADVASU, Ludhiana for postmortem examination. On external examination, brisket edema and enlargement of the prescapular lymph nodes were observed. Grossly, there was traumatic reticulo-pericarditis with suppuration, hepatic congestion and punched-out ulcers in the abomasum. Histopathologically, the heart showed myocardial fibrosis the abomasum revealed lymphoplasmacytic ulcerative abomasitis, the lymph nodes exhibited lymphadenitis and the liver showed features of chronic venous congestion. Blood smear examination revealed the presence of Theileria piroplasms within the red blood cells.

Keywords: Cow, fibrosis, *Theileria*, TRP, ulcer

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Traumatic reticulo-pericarditis (TRP), also known as hardware disease is a condition caused by the ingestion of sharp metallic objects such as nails and pieces of wire[1,2,3]. These foreign objects penetrate the reticulum and can damage nearby anatomical structures including the liver, diaphragm, spleen and heart. The ingestion of such objects is common in cattle grazing in areas where fodder is grown near industrial sites. TRP is a painful and progressive condition that can ultimately lead to the animal's death and results in significant economic losses due to decreased productivity[2,4]. Theileriosis is a tick-borne febrile disease caused by protozoan parasites such as *Theileria annulata*, *T. parva*, *T. lawrencei* and *T. orientalis*, all of which contribute to production losses, morbidity and mortality in cattle[5,6,7]. It affects approximately 39 million crossbred cattle in India, causing substantial economic losses annually[8]. Transmission is primarily mediated by hard ticks belonging to the genera *Hyalomma* and *Rhipicephalus*[5,8]. Theileria has a complex life cycle that requires two hosts. During tick feeding, sporozoites are inoculated into the host and are subsequently taken up by mononuclear cells (MNCs), such as lymphocytes and macrophages. Inside lymphocytes, sporozoites undergo schizogony to produce schizonts (Koch blue bodies)[5,7]. The infection of T-lymphocytes by Theileria reprograms the host cells by upregulating the NF-κB pathway, leading to the expression of anti-apoptotic genes and promoting cell survival[9]. These transformed, proliferating lymphocytes cause lymph node enlargement. Merozoites released from infected lymphocytes then invade red blood cells, causing hemolysis that leads to severe anemia and jaundice[10].

A 4-year-old female Holstein Friesian (HF) cow was presented to the Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab with history of respiratory distress and high fever (104^{O}F) persisting for six days. The animal suddenly collapsed during clinical examination. Bilaterally enlarged prescapular lymph nodes were noted and the animal exhibited abduction of the forelimbs while walking. At necropsy, marked subcutaneous ventral edema was observed in the brisket region. In the abdominal cavity, the abomasum was adhered to the diaphragm. Within the thoracic cavity, the heart was enlarged to approximately three times its normal size and showed adhesions to the ribs. The pericardial sac was markedly thickened and contained approximately 500 ml of yellowish, turbid, fibrino-suppurative fluid. A fibrino-purulent, cheesy mass was adhered to the epicardium, giving the classical "bread and butter" appearance (Fig. 1). A 12-cm long metallic wire was discovered in the reticulum, piercing through the diaphragm into the heart, forming a fistulous tract. The needle's migration tract was discoloured black. Liver and spleen were enlarged; the liver was notably icteric. The liver's surface showed congestion and widespread petechiae. On cut section, the liver exhibited a prominent reticular pattern, imparting a characteristic "nutmeg" appearance (Fig. 2). The prescapular lymph nodes were enlarged, edematous and hemorrhagic (Fig. 3). The abomasum showed severe multifocal punched out ulcerations with associated edema and hyperemia (Fig. 4).

Microscopically, the

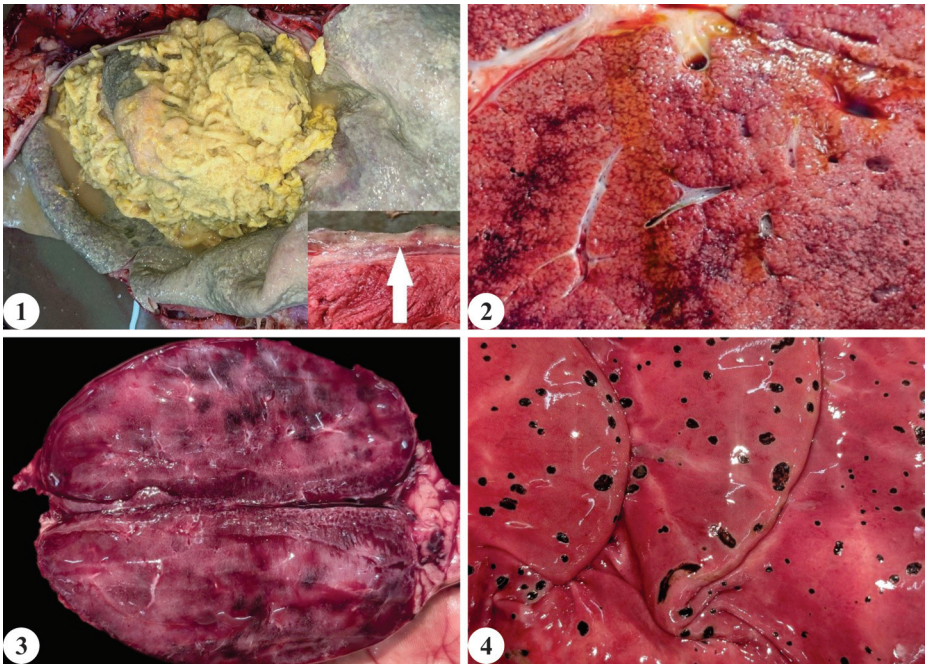

Fig. 1. The pericardial sac covered with fibrin giving bread butter appearance. Inset: Wide band of fibrosis on the surface of the epicardium (arrow); **Fig. 2.** Chronic passive congestion liver cut section shows enhanced lobular pattern; **Fig. 3.** Multiple punched out mucosal ulcers in abomasum with areas of haemorrhages; **Fig. 4.** Oedematous prescapular lymph nodes associated with multifocal coalescing petechiae and ecchymosis.

sections of heart revealed extensive epicardial fibrosis accompanied by suppurative inflammation (Fig. 5). Myocardial cells displayed degenerative changes such as sarcoplasmic swelling, eosinophilia, vacuolation and multifocal lymphoplasmacytic infiltration. The sections of liver showed chronic passive congestion, evidenced by centrilobular hepatic atrophy and periportal sinusoidal congestion (Fig. 6). The section of prescapular lymph nodes exhibited follicular hyperplasia with fibrin, hemorrhage and mononuclear cells within

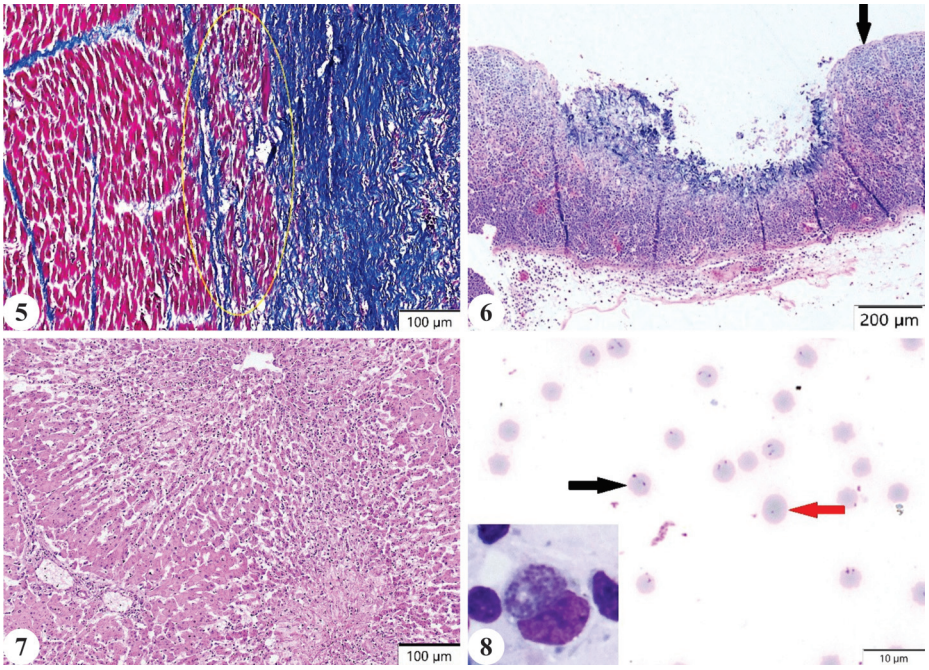

Fig. 5. Heart showing organized granulation tissue having extensive fibrosis (Masson's trichrome stain x100); **Fig. 6.** Focal mucosal abomasal ulcer with raised edges due to proliferation of lymphocytes (arrow) and central area of haemorrhage (H&E x40); **Fig. 7.** Liver shows centrilobular bridging necrosis (H&E x100); **Fig. 8.** Blood smear showing single (red arrow) and multiple (black arrow) *Theileria* piroplasm inside RBCs. Inset: Koch blue body inside lymphocyte pushing the nucleus (Giemsa stain x1000).

the subcapsular sinus. Impression smears from lymph nodes showed numerous schizonts (Koch blue bodies) indenting the nuclei of lymphoblasts. In the sections of the spleen, proliferation and infiltration of macrophages, plasma cells and lymphocytes were evident. The sections of abomasum showed lymphoplasmacytic infiltration in both the mucosa and submucosa with multifocal ulceration of the mucosal surface (Fig. 7). Blood smear prepared from heart blood revealed numerous *Theileria's* piroplasms within many red blood cells, ranging from one to three piroplasms per RBC (Fig. 8).

The pathogenesis of TRP involves the migration of pyogenic bacteria through a fistulous tract created by a sharp foreign object (e.g., needle), originating from the reticulum and extending to the heart. This leads to fibrino-purulent pericarditis characterized by yellowish-green villous projections, imparting a shaggy heart or "bread and butter" appearance[2,3,11,13]. Chronic constrictive pericarditis develops due to organization and fibrosis of the exudate, resulting in thick fibrous connective tissue bands, as observed in the present case (Fig. 1). The constrictive pericarditis, combined with cardiac tamponade due to excessive purulent fluid, contributed to the animal's death[13]. A generalized pathological change in TRP is chronic venous congestion, which causes persistent hypoxia in centrilobular regions of the liver, leading to hepatocellular atrophy, degeneration and necrosis giving rise to the characteristic "nutmeg liver" appearance (Fig. 2).

Anemia and jaundice are common clinical signs in cattle affected by *Theileria* infection, both of which were observed in this case[5,7,10,12]. Prominent pathological findings in Theileriosis include splenomegaly, generalized lymphadenomegaly, abomasal edema and ulceration, edema, epicardial haemorrhages, diarrhoea and/or constipation, hepatic congestion and petechiation on liver[1,9,10]. In this case, jaundice was likely due to a combination of widespread hemorrhages and centrilobular hepatic necrosis resulting from both Theileria schizont infection and chronic venous congestion.

Based on gross and histopathological findings, the present case was diagnosed as a concurrent case of TRP and Theileriosis in a Holstein Friesian cow.

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Conflicts of Interest: None

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CASE REPORT

Capillary haemangioma: Histopathological characterization of a rare gingival mass in a calf

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ABSTRACT

A two months old cross bred Jersey calf was presented with an irregularly oval growth (2.5 cm x 5 mm) in the mandibular gingiva. The mass was greyish-white and ulcerated. The growth was surgically removed and submitted for histopathology. Tissue sample was processed as per standard histological techniques. Microscopically, tissue revealed extensive superficial ulceration with necrosis and bacterial colonies. Underneath, abnormal proliferation of vascular endothelial cells was evident showing plumpy cells with mild anioscytosis, anisokaryosis and low mitotic figures. Deeper tissue revealed abnormal proliferation of vascular capillary network which were separated into lobules by meagre collagenous stroma. Interlobular stroma had loose collagen. Mast cells were scattered in the stromal components of the neoplastic capillary networks. This paper reports the histopathological features of rare case of capillary haemangioma in a two months old cross bred Jersey calf exhibiting multifocal organization of thrombi with recanalization and activity of mast cells in capillary haemangioma.

Keywords: Calf, capillary hemangioma, gingiva, mast cells

Haemangiomas are benign vascular tumours that arise due to abnormal proliferation of blood vessels[1]. Congenital haemangioma is common in children[2] and infrequent in animals[3]. Haemangioma is common in dogs and rare in other domestic animals[4]. Capillary haemangioma is one among the variant of haemangiomas which occur in various organs[5]. Gingival capillary haemangioma is infrequent in calves[6]. Hereditary involvement with mutation in the GNAQ gene is proposed rather environmental cause for haemangiomas[7]. Histologically, multifocal organization of thrombi with florid intravascular proliferation of endothelial cells is a feature which underpin the subject of GNA mutations in haemangioma. Further, mast cells play crucial role as 'double-edged sword' in progression and regression of haemangiomas[8]. Quantity of activated mast cells in stromal cells of haemangioma decide the phase of the tumour[9]. This paper documents the rare gingival capillary haemangioma in a two-months-old crossbred Jersey calf with histopathological features of multifocal well-organized thrombus, exhibiting recanalization and activity of mast cells in stroma of tumour tissue.

A Jersey cross bred calf of two-months-old was presented to Veterinary Clinical Complex, Veterinary College and Research Institute (VCRI), Tamil Nadu Veterinary and Animal Sciences University, Orathanadu, Tamil Nadu with the history of growth in the gingiva of mandibular incisors. The growth was well-defined, whitish-grey and nodular. Surface showed ulceration and had focal area of haemorrhage. The mass was slightly firmer and attached with broad base over the mucosa of labial surface of gingiva of the mandibular incisors. The mass was roughly oval and measured 2.5 cm (length) x 5 mm (width) (Fig. 1). The mass was surgically removed. The tissue was fixed in 10% formalin and submitted for histopathological diagnosis to the Department of Veterinary Pathology, VCRI, Orathanadu. Biopsy tissue was processed as per standard paraffin-embedding technique[10]. Tissue sections of 3-4 µm thickness were prepared and stained as per haematoxylin-eosin staining protocol[11]. Duplicate sections were stained with Masson trichrome stain[12], standard toluidine blue stain[13] and Heidenhain's hematoxylin[11].

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Microscopically, the tumour mass was dome-shaped (Fig. 2) showing extensive superficial ulceration of the mucosa, necrosis with bacterial colonies and a focal area of haemorrhage. A focal area of the mucosa with stratified squamous epithelium was evident towards lower-side of the tissue. Ulcerated areas revealed throngs of infiltrative neutrophils extending a little deep into the dermis (Fig. 3). Underneath, the tissue was highly cellular and revealed the proliferative vascular endothelial cells forming numerous variable sized channels filled with erythrocytes (Fig. 4). The vascular lining cells were

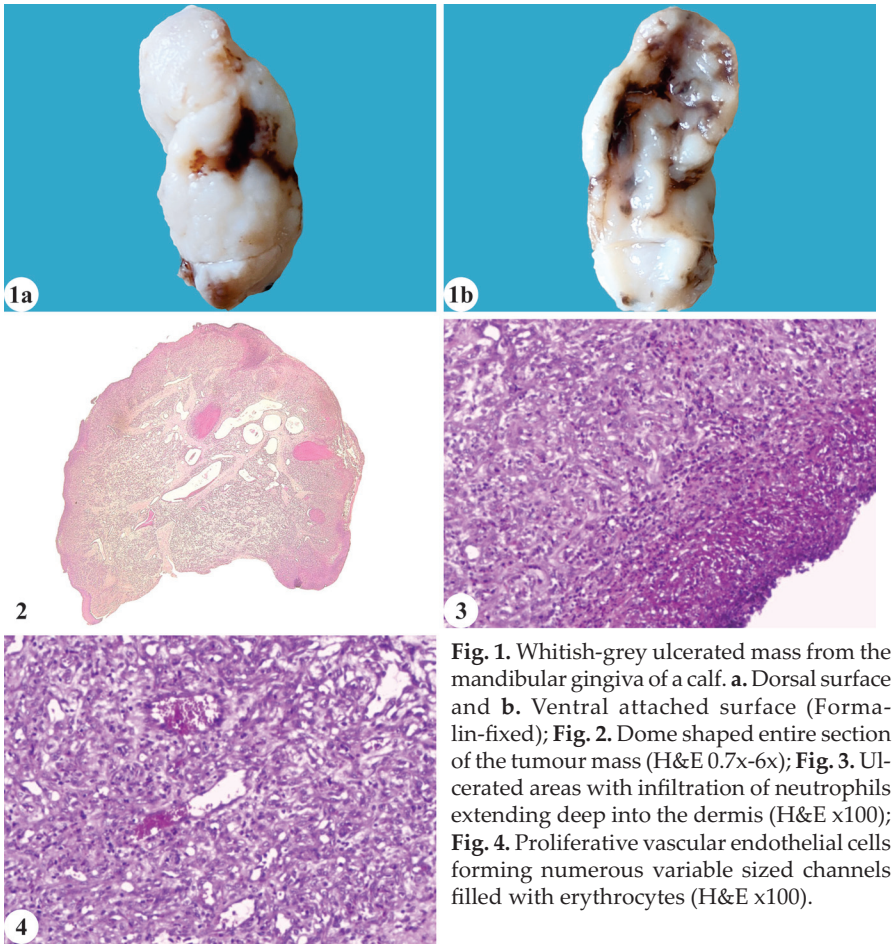

Fig. 1. Whitish-grey ulcerated mass from the mandibular gingiva of a calf. **a.** Dorsal surface and **b.** Ventral attached surface (Formalin-fixed); **Fig. 2.** Dome shaped entire section of the tumour mass (H&E 0.7x-6x); **Fig. 3.** Ulcerated areas with infiltration of neutrophils extending deep into the dermis (H&E x100); **Fig. 4.** Proliferative vascular endothelial cells forming numerous variable sized channels filled with erythrocytes (H&E x100).

rotund. These endothelial cells found to infiltrate deep into the dermis. In the transit area, the cells were corpulent and showed slight pleomorphism (Fig. 5a). These cells had prominent ovoid to spindle shaped vesicular nucleus with stippled chromatin (salt and pepper chromatin pattern) (Fig. 5b) and had very low mitotic figures (0-4/10 high power fields x400). Cells revealed mild anisocytosis and anisokaryosis. Transit area also revealed abnormal capillaries with narrow and or wider luminal area. These capillaries were either filled with blood or serous fluid. Few of these capillaries showed well-formed organized thrombus (Fig. 6). The thrombus had proliferation of endothelial cells into the thrombotic mass (Fig. 7).

Deep in the dermis, the neoplastic endothelial cells formed lobules of irregularly arranged, variable sized, closely packed vascular network. The lobules were separated by moderate collagenous stroma (Fig. 8). Vascular clusters showed irregularly anastomosing thin walled capillaries with uneven lumen (Fig. 9). Capillaries were lined by single layer of plumpy endothelial cells (Fig. 10). Lumen of the neoplastic capillaries either contained few erythrocytes or mostly empty. Some capillaries showed scattered and indistinct lumina. Lobules of capillaries were separated by moderate amount of collagen which was evidenced on Masson trichrome stain (Fig. 11). Stroma between the capillaries contained loose, thin collagenous stroma (Fig. 12). The stroma contained scattered mast cells and plasma cells. Mast cells (MCs) were lesser in the superficial portion and scattered in the stromal elements of the deeper neoplastic capillary network (Fig. 13).

Vascular anomalies were categorized into (a) Congenital vascular malformations (hamartoma) and (b) Neoplastic transformations. Vascular neoplastic transformations (angioma) are classified into benign haemangioma and malignant haemangiosarcoma[14]. Further haemangioma are sorted into capillary, cavernous and epithelioid haemangioma[14] on the basis of the size of the vascular channels[15] and appearance of endothelial cells respectively[16,17].

Haemangiomas were found to be less frequent in animals[18]. It was reported to occur as cutaneous tumour in skin[15,19], subcutis[3] and visceral tumour in internal organs[16]. The occurrence of haemangioma was rare and infrequent in oral cavity[20]. It was reported from the tongue of a dog[21]. Vascular tumours of gums were found to be rare in animals[22] and extremely rare in calves[20]. A unique report of haemangiosarcoma of gingival tissue

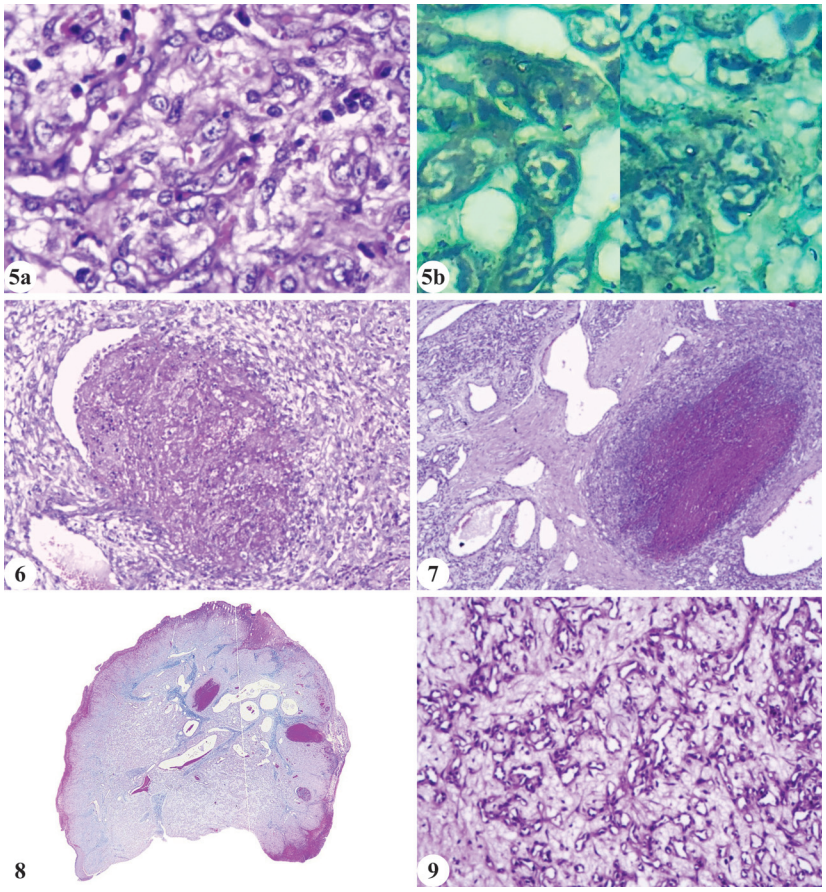

Fig. 5a. Plumpy neoplastic vascular endothelial cells with prominent ovoid to spindle shaped vesicular nucleus with stippled chromatin (H&E x400); **Fig. 5b.** Stippled chromatin (resembling salt and pepper) of proliferating vascular endothelial cells (Heidenhain's hematoxylin with light green counter stain x1000); **Fig. 6.** A neoplastic capillary with well-formed organized thrombus; **Fig. 7.** Organised thrombus exhibiting proliferation of endothelial cells into the thrombotic mass (H&E x40); **Fig. 8.** Neoplastic mass shows meagre collagen in entire tumour mass (Masson trichrome stain 0.7x-6x; **Fig. 9.** Irregularly arranged, variable sized, closely packed capillary network separated by collagenous stroma (H&E x100).

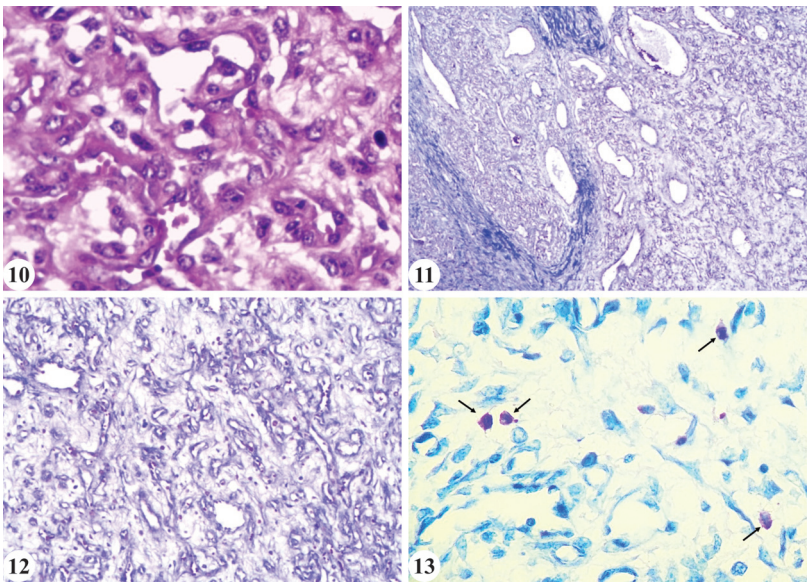

Fig. 10. Neoplastic capillaries are lined by single layer of plumpy endothelial cells (H&E x400); **Fig. 11.** Neoplastic mass shows separation of lobules with collagen bundles (Masson trichrome stain x40); **Fig. 12.** Irregular capillary network separated by loose collagenous stroma (Masson trichrome stain x100); **Fig. 13.** Scattered mast cells (arrow) in stromal tissue of capillary haemangioma (Standard toluidine blue stain x400).

was reported in a 2-months-old calf of native breed in Iran[20].

In the catalog of vascular tumours, the capillary haemangioma of gingiva was found to be uncommon in animals[6,17,23,24]. Infrequently, capillary haemangioma was described in various organs/tissues in animals[4,5,19,25]. The earlier reports documented the glossal capillary haemangioma in bovine[6,23,24,26] and cat[4].

Histopatholgical findings described in the present study was similar with the previous reports[6,19,21,23,24,27]. The feature of capillary thrombus with recanalization recorded in the study was in-line with previous reports[7,20]. Multifocal vascular thrombus, organization of thrombi with intravascular proliferation of endothelial cells, which were streaming into the thrombi were the distinctive features. These changes were observed immediate underneath the ulcerated area and in the transit zone composed of proliferative neoplastic cells.

The more remarkable feature was the presence of scattered mast cells (MCs) in the stroma of the neoplastic vascular stroma. These cells were lesser in superficial areas rather moderate in the deeper neoplastic tissue. Evidence of mast cells and plasma cells in the stroma of deeper neoplastic tissue of the present study might not indicate the inflammatory reaction. However, in contradictory to the observation of[19] have described the inflammatory lobular capillary haemangioma in the skin of left ventral neck of guniea pig[19]. It was proposed that MCs plays pivotal role in progression and regression of vascular tumours[28]. It was thought that MCs were found to be low in proliferating phase, increase in early involution phase and later on decrease in late involution phase of the haemangiomas[9]. Biological role of these MCs in haemangioma was studied by[8]. Activated MCs secrete both angiogenic and anti-angiogenic modulators in haemangioma. Among, Type III collagen and fibroblast growth factor (FGF-2) hold the key in endothelial cell proliferation during proliferation of haemangioma, whereas interferons (INF-α, β and γ) downregulate the FGF-2, endothelial cell proliferation and inhibit mitogenesis of vascular endothelial growth factor (VEGF) respectively[8]. Low number of MCs in the superficial lesion with abundant endothelial cells might indicate the proliferative phase of the tumor. The presence of scattered MCs in the stromal capillary network in deeper lesion might possibly reflect the involution phase of the haemangioma in the present study. To summarize, MCs were regarded as paradoxical in vascular tumours as documented earlier[8,9].

Elaborative studies on the role of MCs in haemangioma is imperative for better understanding on the biology of MCs in vascular tumours of animals. This report records the incidence of gingival capillary haemangioma with thrombotic features in a two months old cross bred Jersey calf.

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CASE REPORT

A rare case of Aspergillosis in a goat

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ABSTRACT

A goat of 2 years was presented for necropsy examination. Postmortem examination of the animal revealed lungs with multiple firm creamish nodules which were observed in both the lungs. Histopathological examination of lungs revealed a typical pyogranulomatous reaction with fungal hyphae in a central necrotic area. The hyphae were demonstrated by Grocott's and PAS staining. Cultural examination revealed bluish-green colonies on Sabouraud dextrose agar, indicating Aspergillosis. Lactophenol cotton blue staining revealed conical heads with a uniseriate row of phialides on the upper two-thirds of the vesicle.

Keywords: Aspergillosis, goat, lungs, pneumonia

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In India, pneumonia is the biggest cause affecting both organized and nomadic small ruminant flocks. It is the cause of death in goats of all ages, with mortality being more in young kids. According to one study there was 22.8% mortality in goats due to pneumonia in an organised farm[1], the numbers can be much high in unorganised sector. Pneumonia is best understood as a disease complex that involves interactions between the host (physiological and immunological), many agents (bacterial, viral and fungal) and environmental variables. When invasive microorganisms reach a threshold dosage compared to host sensitivity, non-specific defence systems are activated and proliferation takes place, compromising the lung's defence mechanisms. The hallmark of pneumonia is inflammation of the pulmonary parenchyma and surrounding bronchioles[2]. Although fungus infections can occur in healthy animals, they are more frequently opportunistic infections in immunocompromised and disabled hosts with reduced natural defences. Mostly it leads to deadly consequence since the fungal infection can go undetected[3]. The primary source of mycotic infections is spore inhalation, which can result in hemo-lymphatic spread. The primary cause of mycotic pneumonia has been identified as Aspergillus species, *Cryptococcus neoformans*, *Pseudoallescheria boydii* and Candida species[4,5].

A two-year-old male carcass with a history of respiratory trouble, dyspnea, and a moderate fever before death was brought to the Postmortem Hall, Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. A thorough necropsy was performed. Swabs from the lungs were taken aseptically, processed and cultured on Sabouraud dextrose agar (SDA). For mycological analysis, the plates were inverted and incubated for a week at 25 to 35^{O}Celsius. Using a drop of lactophenol cotton blude stain (LPCB), the fungal growth observed on SDA was teased on the clean glass side, covered with glass slip and under a light microscope[6]. Tissue sample from lungs were collected in 10% neutral buffered formalin as per the method described[7]. 4 µm tissue sections were cut using a semi-automatic microtome (Leica, Germany) and stained with hematoxylin and eosin (H&E)[7]. Grocott and Periodic Acid Schiff (PAS) staining was used to duplicate paraffin slices to reveal the presence of bacterial or fungal etiological agents[8].

Macroscopic lesion of lungs showed multifocal, often exhibiting purple-consolidated regions with white or cream colour nodules varied from 2-4 cm containing semi-liquid white or cream colour pus (Fig. 1). Multiple firm, cream to yellow-colored granulomas that ranged in diameter from a few millimeters to several cm (5 mm to 2 cm) were seen throughout the consolidated lungs. Similar findings were reported in lungs of goat and sheep[1]. Blue-green to grey mould colonies with suede-like surface consisting of conidiophores on SDA plates were observed (Fig. 2A), indicating *Aspergillus*. The fungal colonies of Aspergillus on staining by LPCB stain revealed conidial heads with a uniseriate row of phialides on the upper two-thirds of the vesicle (Fig. 2B) in agreement with earlier study[9]. On histological examination, the lung revealed pyogranulomas (Fig. 3A), core necrotic regions with a large no. of neutrophils and eosinophils and the epithelioid macrophages, lymphocytes and plasma cells that surround them. Aspergillus species have slender hyphae (Fig. 3B) that vary in

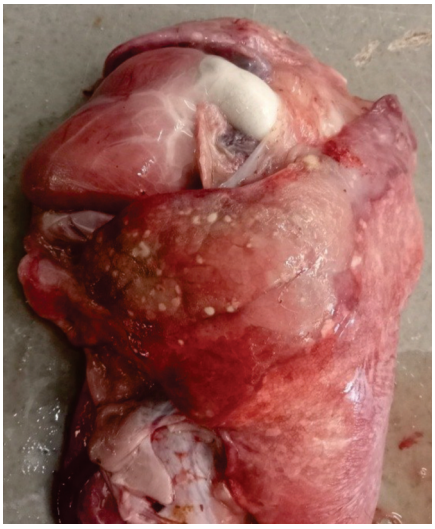

Fig. 1. Lung surface showing cream coloured, multiple, firm, granular nodules of different size.

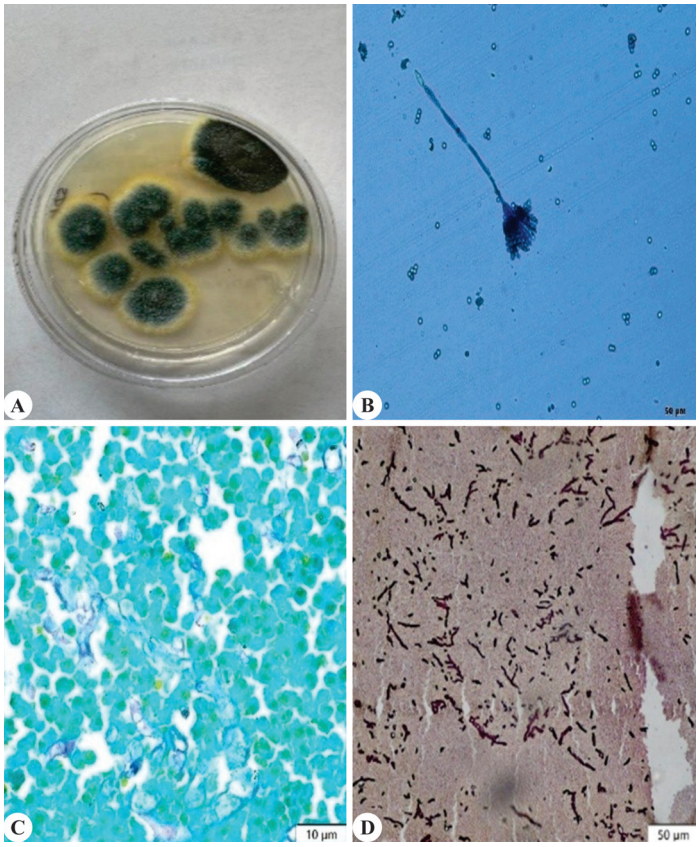

Fig. 2A. Showing isolation of fungus on SDA agar Blue-green, powdery and pale yellow on the reverse. **B.** Lactophenol cotton blue staining showing the spores of fungus, suggestive of *Aspergillus* (LPCB x50 µm). **C.** Lung showing unstained fungal hyphae (Periodic acid-Schiff x10 µm). **D.** Lung showing black colour fungal hyphae (Grocott methenamine silver x50 µm).

width from 3 to 12 µm. They may be identified by their distinct separation, dichotomous branching pattern and parallel edges and it has been confirmed by periodic acid-Schiff[10] (Fig. 2C) and Grocott methenamine silver (GMS) stain method (Fig. 2D). Histopathological analysis of the lung tissue of infected sheep and goats revealed that the hallmark of mycotic pneumonia was the presence of multiple focal mycotic granulomas, septate hyphae of Aspergillus scattered in the centre of the granuloma, surrounded by polymorphonuclear cells, lymphocytes and macrophages, as well as proliferation of fibrous connective tissue[11]. The present findings in this study were in accordance with previous observations of[1,12,13].

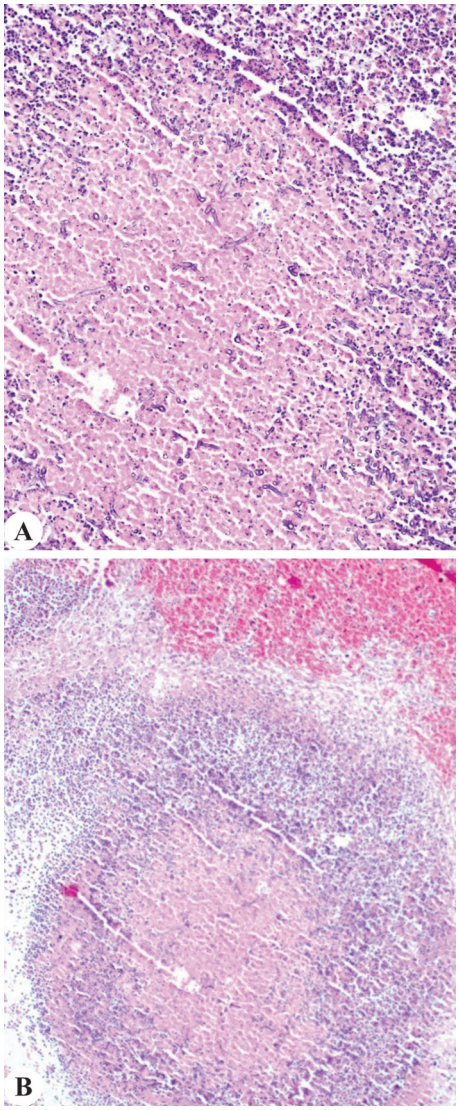

Fig. 3A. Lung showing granuloma formation with a necrotic centre surrounded by polymorphonuclear cells (H&E x100 µm). **B.** Lung showing granuloma formation with a necrotic centre containing fungal hyphae surrounded by polymorphonuclear cells especially neutrophils macrophages, lymphocytes and multinucleated giant cells (H&E x50 µm).

CONCLUSION

The present case was diagnosed as Aspergillosis in goat after taking into consideration of gross lesions,

histopathological examination, special staining and cultural findings.

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CASE REPORT

A Case of Soft Tissue Fibroma in Wistar Rat *(Rattusnorvegicus)*

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ABSTRACT

Fibromas are benign connective tissue tumours arising from fibroblasts and are occasionally observed as spontaneous neoplasms in laboratory animals. In laboratory rats *(Rattusnorvegicus)*, fibromas are considered uncommon but may occur in aging individuals, often as incidental findings during long-term toxicity or carcinogenicity studies. Present clinical case report management of a fibroma in an 18-month-old female wistar rat. The animal presented with a firm, slow-growing, non-ulcerated mass located on the dorsal thoracic region. Clinical examination revealed hard mass in thoracic region with problem in normal feeding. The mass was surgically excised and subjected to histopathological evaluation, which showed a well-circumscribed proliferation of mature fibroblasts arranged in interwoven bundles within a dense collagenous matrix, with no evidence of mitotic activity, nuclear atypia or tissue invasion. Based on these findings, a diagnosis of fibroma was confirmed.

Keywords: Fibroblast, fibroma, histopathology, mitoticactivity, thoracic region

Neoplastic diseases in laboratory animals have long been a subject of interest in both basic and applied biomedical research. Rodents, especially rats and mice are widely used in toxicological studies due to their genetic uniformity, relatively short lifespan and well-documented background lesion profiles[1]. Among these, soft tissue tumours such as fibromas are considered relatively rare but important, especially when evaluating chronic toxicity or carcinogenicity studies[2]. Fibromas are benign mesenchymal tumours composed primarily of mature fibroblasts and dense collagenous stroma, typically presenting as well-demarcated, non-invasive masses in subcutaneous tissues[3]. While fibromas are more frequently encountered in domestic animals, their occurrence in laboratory rodents especially rats *(Rattusnorvegicus)* is sporadic and generally limited to older animals or those involved in long-term studies[4]. In a retrospective analysis of 940 untreated Hannover Wistar rats (RjHan:WI), subcutaneous fibroma was observed in 4.7% of males and 3.6% of females while major neoplasms overall were most frequent in the endocrine, integumentary and reproductive systems. Fibroma was among the common mesenchymal tumours, alongside fibrosarcoma and thyroid C-cell adenoma[5]. In rats, fibromas most commonly arise in the subcutis of the trunk or limbs and are generally asymptomatic unless they reach a size that causes mechanical interference or ulceration[6]. The incidence may vary among rat strains and is often higher in aged males, particularly under prolonged housing conditions[1]. Differentiation of fibromas from other spindle-cell tumours, such as fibrosarcomas, is critical due to differences in biological behaviour and prognostic implications. While fibromas are typically non-invasive and lack mitotic activity, fibrosarcomas exhibit cellular atypia, infiltrative growth and frequent mitotic figures, necessitating careful histopathological assessment for accurate diagnosis[2,3]. This distinction is especially important in regulatory toxicology, where the nature of neoplastic lesions can influence the classification of a substance's carcinogenic potential[7]. This case report describes a spontaneous subcutaneous fibroma in an adult female wistar rat. The report aims to document the clinical, gross and histopathological features of this uncommon lesion, emphasizing the importance of distinguishing spontaneous background pathology from treatment-related effects in laboratory animal studies.

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An 18-month-old female wistar rat, weighing approximately 480 grams at the time of lesion detection, was presented to the Teaching Veterinary Clinical Complex, Bihar Veterinary College, Patna with a history of a slowly growing lump in the dorsal thoracic region, progressing over the past three months (Fig. 1). Clinical signs observed included shallow breathing, loss of appetite, drooling of saliva and weight loss. Further examination revealed that the animal appeared to be in pain and had a hard, palpable mass in the thoracic region of size 10.2 cm (Fig. 2). Based on these clinical findings, the lesion was tentatively diagnosed as a neoplasm and surgical excision was planned. Surgery was performed under general anaesthesia. The animal was premedicated with butorphanol at a dose rate of 0.1 mg/kg body

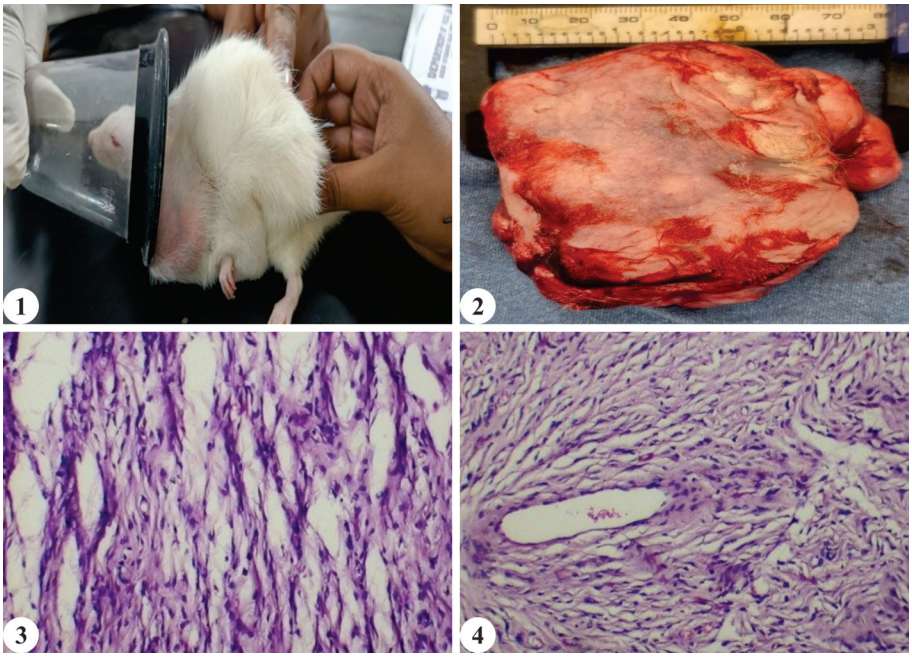

Fig. 1. Female Wistar Rat showing a prominent subcutaneous abdominal mass suggestive of a tumorous growth. The rat is being anesthetized using an induction chamber. The mass is hard in consistency and is in the ventral abdominal region; **Fig. 2.** Excised subcutaneous tumour mass of 10.2 cm from a rat. The mass is encapsulated with an irregular surface and prominent vascularisation; **Fig. 3.** Histological section of a fibroma of a rat (H&E 10x) showing a well-differentiated proliferation of spindle-shaped fibroblastic cells arranged in interwoven bundles within a dense collagenous stroma; **Fig. 4.** Histological section of a fibroma from a rat (H&E 10x) showing a well-circumscribed, hypocellular mass composed of spindle cells aligned in a storiform and fascicular pattern.

weight subcutaneously, followed by induction and maintenance with sevoflurane. An elliptical incusion was made around the mass and the tumorous tissue was excised from its base. Haemorrhage was controlled by ligating the blood vessels using polyglactin 910 (Vicryl) No. 3. The excised tumour mass was submitted for histopathological examination. The skin incusion was closed routinely using simple interrupted sutures.

Postoperatively, the animal was treated with syrup meloxicam at a dose of 2 mg/kg body weight on the first day, followed by 1 mg/kg body weight for the next two days. Additionally, cefpodoxime was administered orally at a dose of 15 mg/kg body weight for five days. The owner was advised to perform regular wound dressing.

Histopathological evaluation of the subcutaneous mass revealed classical features of a benign fibrous tumour. The lesion was composed predominantly of interlacing bundles of spindle-shaped fibroblasts, exhibiting uniform morphology with elongated, cigar-shaped nuclei and indistinct cell borders (Fig. 3). These cells were embedded in a dense, eosinophilic collagen matrix, which imparted a whorled appearance on low magnification. The tumour was well circumscribed and encapsulated (composed of collagen fibres, fibroblast and inflammatory cells) with no evidence of infiltration into adjacent skeletal muscle or dermis. The fibroblasts exhibited minimal pleomorphism and mitotic figures were extremely rare (<1 per 10 high-power fields), suggesting low proliferative activity. There was a clear absence of features consistent with fibrosarcoma or other soft tissue sarcomas namely, cellular atypia, increased mitotic activity, invasive growth patterns and poorly defined margins[2]. These findings support the benign nature of the lesion and validate the histological diagnosis of a subcutaneous fibroma.

Fibromas are benign tumours originating from fibroblasts, the principal cells of connective tissue and are characterized by slow growth and non-invasive behaviour[3]. Although spontaneous fibromas are common in some domestic animals, their occurrence in laboratory rodents is rare. Reported prevalence rates vary by strain and study but generally range from approximately 1% to 5% in control populations, with Wistar and Sprague-Dawley rats showing fibroma incidences of around 1% to 4.7% in historical control data[5,8]. When they do occur, they are typically incidental findings during chronic studies in aged rats, particularly in control groups or those exposed to inert test substances[4]. Estrogen plays a crucial role in promoting the growth of fibrous tissues by stimulating the proliferation of fibroblasts and other connective tissue cells, leading to the development of fibromas, while progesterone regulates tumour growth by promoting differentiation over proliferation; however, the balance between estrogen and progesterone is vital for tissue homeostasis and when disrupted, such as by

chronic estrogen exposure or low progesterone levels, fibromas can form[9]. Fibromas in female rats may be associated with ovarian dysfunction, which can lead to hormonal imbalances that contribute to the development of fibromas[10]. In this case, the fibroma developed in an 18-month-old female rat. The absence of any treatment-related exposure supports the classification of this lesion as spontaneous in origin. Age is a known contributing factor in the development of spontaneous tumours in laboratory animals and fibroblastic proliferations are more likely to occur with advancing age[1]. The histopathological characteristics observed well-demarcated margins, low cellularity and absence of mitoses are consistent with previously published descriptions of rodent fibromas[2]. These features not only differentiate fibroma from fibrosarcoma but also from other mesenchymal tumours such as leiomyoma, lipomas or peripheral nerve sheath tumours. Although fibromas are benign, their clinical significance in laboratory studies lies in their potential to confound results, particularly if they become large, ulcerate or interfere with mobility or feeding. In this case, the tumour was localized, asymptomatic and affecting the overall health or behaviour of the animal.

CONCLUSION

This case report describes a rare instance of a spontaneous subcutaneous fibroma in an aged female rat. The lesion was well-circumscribed, composed of mature fibroblasts within a dense collagen matrix and showed no evidence of malignancy or invasive behaviour. The absence of treatment-related exposure and the animal's advanced age support the classification of the tumour as a spontaneous lesion. While fibromas are uncommon in laboratory rats, their recognition is essential for accurate pathological assessment and the differentiation of incidental findings from potential compound-related effects in toxicological studies. This case emphasizes the shared medical considerations for rats as both research models and pets.

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Title of Thesis	:	Pathology of Lymphoid Leukosis in chickens
Name of the Student	:	**Charlee Porte**
Name of the Advisor	:	Dr D.K. Jolhe
Degree/Year	:	MVSc/2022
Name of the University	:	Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Anjora, Durg, C.G.

The present study was undertaken to know the prevalence, pathology and molecular diagnosis of Lymphoid Leukosis in chickens. The study was conducted on 150 birds presented for post mortem examination to the Department of Veterinary Pathology, College of Veterinary Science and AH, Anjora, Durg, for a period of 6 months from Durg, Rajnandgaon, Balod and Raipur districts of Chhattisgarh. On the basis of necropsy examination 28 birds showed tumourous swelling in visceral organs. Samples were collected from all 28 cases which formed the basis of the present study. Kadaknath, desi and layer poultry flocks of 16 weeks or older age group were found to be affected with Lymphoid Leukosis which showed mortality of 12.19%, 6.89% and 3.22% in Durg, Rajnandgaon and Raipur districts of Chhattisgarh, respectively. The prevalence of lymphoid leukosis in the present study on the basis of necropsy examination and RT-PCR was found to be 18.6% and 8.6%, respectively. The affected birds showed anorexia, emaciation with prominent keel bones, weakness and diarrhoea. Enlargement of liver can be detected by palpation of abdomen. Some birds showed pale, shriveled and cyanotic comb. Grossly, tumours were found in liver, spleen and kidneys. Liver was involved in 28 cases. Liver of kadaknath and desi birds were having 3 types of tumorous growth i.e. nodular, granular or milliary and diffuse. Marked diffuse enlargement of liver is observed in most cases, which occupied the whole abdominal cavity. Visible tumours were found on the liver surface which were soft, smooth and glistening, cut surface showed greyish to creamy white coloured areas of necrosis and raised tumorous nodules. Spleen of desi birds were involved in 24 cases and were severely congested with marked enlargement. It is almost double the size of normal spleen and some petechial haemorrhages were also seen. Cut surface of spleen was very soft, pulpy, nodular and greyish to creamy white in colour. Kidneys of desi birds were involved in 18 cases and showed enlargement and congestion with well demarcated lobules. Lungs were congested with multiple nodules and consolidated in most cases. No gross lesions could be observed in any of the bursa of birds examined.

Histopathologically, the section of liver revealed diffuse and focal aggregates of proliferating uniform sized immature lymphoid cells which displaced and compressed the hepatocytes causing degeneration and necrosis of adjacent hepatocyte. Sinusidal spaces were narrowed and infiltrated with lymphoblasts. Neovascularization was also observed. Severe lymphoid cell proliferation around the blood vessels and degenerative and necrotic changes in the adjacent hepatocytes were also observed. Proliferation of uniform lymphoid cells around the blood vessels imitating early metastasis. Foci of tumourous lymphoid cells with individualization of hepatocytes was also noticed. Spleen of affected birds showed depletion of lymphocytes in and around germinal centre. Spleen exhibiting increased tumour cells destroying normal arrangement of splenic pulp. Proliferating neoplastic lymphoid cells were found in the sections of spleen. Kidney of ALV infected birds showed diffuse infiltration of uniform lymphoid cells in interstitial spaces and degeneration and atrophy of glomeruli. Swollen and degenerated tubular epithelial cells and severe infiltration of uniform lymphoid cells in the interstitium. Infiltration of uniform lymphoid cells in the interstitium with atrophic glomeruli. Lungs depicted neovascularization and nodular aggregates of proliferating uniform sized lymphoid cells which displaced and compressed the parenchymal tissues of the organ. Lymphoid aggregates in the parenchyma were found with central necrosis. No microscopic lesions could be detected in the bursa of ALV infected birds. Out of 150 Lymphoid Leukosis suspected chickens, 28 showing typical postmortem lesions (marked enlargement of liver and spleen with tumorous growth and enlarged and congested kidneys).

Out of 28 samples, 13 samples produced an amplicon of 326 bp confirming overall LL positivity in 8.6% chickens. In Durg district, 10 out of 82 samples (12.19%), in Rajnandgaon district, 2 out of 29 samples (6.89%) and in Raipur district only 1 case out of 31 samples were found to be positive for LL by RT-PCR (3.22%). Thus, it showed Durg district had higher number of LL positive cases by RT-PCR as compared to Rajnandgaon and Raipur districts. The sequencing result showed that only conserved *pol* gene sequence present among the subgroup (A-E) could be amplified by primers (AD1 & H5) and sequenced. The Nucleotide sequence of ALV Durg, India showed 99.68% similarity with Iran (KT326193), Taiwan (HM582658), China 1 (MT783272), USA 2 (MF817823) and USA 1 (MF817822). The nucleotide sequence of the present study showed 99.36% similarity with China 4 (KF575327), China 2 (MK951780), IVRI India 1 (KY933440), IVRI India 2 (KY933431) and China 5 (KF575326) whereas, Japan 1 (AB670317) and Japan 2 (AB670315) showed 99.04% similarity. Phylogenetic analysis showed 13 clades. ALV Durg India formed a cluster with the sequences of Avian leukosis virus, Iran. Therefore, it can be concluded that ALV Durg isolate and the isolates from Iran had a common ancestor.

Title of Thesis : Pathology and molecular identification of respiratory fowl adenovirus infection in broilers

Name of the Student : **Akash Suman**

Name of the Advisor : Dr Nidhi Shrivastava

Degree/Year : MVSc/2024

Name of the University : College of Veterinary Science & AH, Mhow, Nanaji Deshmukh Veterinary Science University, Jabalpur, MP

In the present research, broiler mortalities with respiratory symptoms from 12 farms in the Indore region were examined, focusing on postmortem examination and molecular techniques to detect Fowl adenovirus infections. Clinically broilers displayed signs of lethargy, huddling, facial oedema, reduced feed and water intake and severe respiratory distress, including laboured breathing and nasal discharge.

Postmortem findings revealed gross lesions in the trachea, lungs and liver. Grossly lesions varied from mild congestion to severe haemorrhages and microscopically, deciliation with basophilic intranuclear viral inclusions present in the lungs, lesions included focal emphysema, haemorrhages and red hepatization while liver lesions showed enlargement, congestion and necrosis. Microscopically, intranuclear viral inclusions were observed in both the lungs and liver further indicating adenovirus replication in these organs.

Tissue samples from affected organs were analyzed using polymerase chain reaction targeting the hexon gene for FAdV detection. Out of the 12 farms, 5 tested positive for FAdV with farm 01, 07 and 11 showing widespread infection in the trachea, lungs and liver. Farm 05 and 06 exhibited localized infection in the lungs and liver but negative results in the trachea for FAdV. Despite respiratory involvement, ali nasal swabs tested negative for the virus, likely due to low viral load or insufficient viral concentration, highlighting the limitations of using nasal swabs for diagnosing FAdV infections. The PCR amplification of the hexon gene followed by sequencing, identified FAdV species D serotype 2 as the causative agent in the positive samples.

Title of Thesis : Pathohaematobiochemical study of paratuberculosis in non-target organs of goats *(Caprahircus)*

Name of the Student : **Anil Bittal**

Name of the Advisor : Dr G.P. Jatav

Degree/Year : MVSc/2024

Name of the University : College of Veterinary Science & AH, Mhow, Nanaji Deshmukh Veterinary Science University, Jabalpur, MP

The present study was designed to investigate the incidence of paratuberculosis along with the haemato-biochemical alterations and gross and histopathological changes observed in non-target organs (liver, spleen, kidney, testisandovary) in goats. In the present study, samples were taken from a total of 250 goats that were slaughtered at the Cantonment Board slaughter house in Dr Ambedkar Nagar (Mhow).

Goats from various age and health groups were examined using faecal smear microscopy, impression smears, histopathology and biochemical analysis. The overall MAP incidence was 34.4% based on faecal smear examination. Target organ analysis revealed MAP positivity in 22.8% of intestinal and 19.6% of mesenteric lymph node samples. Non-target organs displayed lower positivity with 2.8% in the liver and 0.8% in the spleen. Kidney, testis and ovary showed no *MAP* presence. The highest incidence was observed in goats with a BCS of +3 (normal). The incidence was higher in adult goats over two years (21.20%), with fewer cases in younger goats.

Haematological analysis indicated reduced haemoglobin, packed cell volume and erythrocyte count in *MAP*-infected goats alongside elevated leukocyte counts. Biochemical findings highlighted decreased total protein and increased liver enzyme activities.

Gross pathology revealed severe intestinal corrugation, enlarged mesenteric lymphnodes with blackened, congested cores and systemic signs in non-target organs. Histopathological observations included cellular infiltration, lymphocyte depletion and hepatocyte degeneration. Acid-fast staining confirmed *MAP* presence in affected tissues.

This study underscores the need for effective diagnostic methods and control programs to mitigate paratuberculosis impact on goat health and productivity, emphasizing its localized nature with sporadic systemic involvement. These findings provide a foundation for future research and targeted disease management strategies.

Title of Thesis : Pathohaematobiochemical studies on paratuberculosis in target organs of goats

Name of the Student : **Shivraj Chouhan**

Name of the Advisor : Dr G.P. Jatav

Degree/Year : MVSc/2024

Name of the University : College of Veterinary Science & AH, Mhow, Nanaji Deshmukh Veterinary Science University, Jabalpur, MP

The present study was designed to study the incidence of paratuberculosis in goats, haematobiochemical changes and gross histostopathological changes in target organs, 250 goats were examined at the cantonment board slaughterhouse in Mhow, Indore. The study focused on both male and female goats presented for slaughter, with a comprehensive sampling strategy that included 250 faecal, blood, serum and tissue samples from the intestine and mesenteric lymph nodes. The overall incidence of paratuberculosis was found to be 30.80% with 77 goats testing positive via faecal smear examination.

Clinical signs observed during antemortem examination included diarrhoea, pasty faeces, emaciation and rough hair coats. Gross lesions indicated thickening and congestion of intestinal tissues, along with enlarged and calcified mesenteric lymph nodes, characteristic of paratuberculosis. Incidence based on direct microscopy of faecal samples using Ziehl Neelsen staining identified 30.80% positivity, while impression smears of target organs revealed acid-fast bacilli in 19.20% of intestinal samples and 15.60% of lymph node samples.

The incidence was higher in adult goats over two years (19.60%), with fewer cases in younger goats. Haematological analysis indicated significant decrease in haemoglobin, packed cell volume and total erythrocyte count in affected goats, while total leukocyte count, lymphocytes and eosinophils were increased. Serum biochemical analysis showed elevated levels of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, with reduced total protein levels in positive cases.

Post-mortem examination revealed characteristic lesions of muscular atrophy, thickened intestines and edematous mesenteric lymphnodes. Histopathological evaluations of intestine and mesenteric lymph nodes indicated extensive inflammatory response, including the presence of microgranulomas and infiltration of mixed population of inflammatory cells consistent with chronic granulomatous inflammation.

Title of Thesis : Pathomorphological and functional studies of pancreas with special reference to digestive and respiratory system in Kadaknath birds

Name of the Student : **Pratiksha Upadhyay**

Name of the Advisor : Dr Supriya Shukla

Degree/Year : MVSc/2024

Name of the University : College of Veterinary Science & AH, Mhow, Nanaji Deshmukh Veterinary Science University, Jabalpur, MP

The present study was designed as the pathomorphological and functional study of pancreas with special reference to digestive and respiratory system in Kadaknath birds. This study was conducted in Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Mhow, MP along with college poultry unit and government and private poultry farms in and around Indore district. In the present investigation, total 230 Kadaknath birds were examined for various digestive and respiratory symptoms. The representative blood samples from 10% diseased live birds (23 samples) were collected for insulin estimation. Total 150 dead Kadaknath birds were surveyed for gross and histopathological study of pancreatic lesions and other related organs.

Blood serum samples were used for evaluation of insulin concentration by ECLIA method of insulin estimation. Total 17 samples had higher insulin concentration than normal value indicative of functional derangement of affected pancreas.

Pancreatic pathology was evaluated through an examination of various parameters following post-mortem assessments. These parameters encompassed an analysis of gross pathology in deceased birds, including a detailed investigation of pancreatic pathology. Histopathological analysis was conducted using routine Hematoxylin and Eosin staining, with lesion confirmation achieved through histochemical examination utilizing different stains such as Heidenhain's Iron Haematoxylin stain, PAS stain and Gomori's staining technique for pancreatic islet cells. Special staining gave negative results with destruction of islets of langerhans.

The birds were seen with clinical signs of both system (septicemia) and higher concentration of insulin along with greater extent of pancreatic lesions in affected dead birds, this establishing a correlation of hyperactivity of pancreas in septicaemia in birds. The incidence of pancreatic lesions was higher in the diseases which affect both systems. It was in colibacillosis (84.61%), pullorum disease (50%), ranikhet disease (80%) and infectious bronchitis (66.66%) respectively.

Title of Thesis : Clinicopathology of hepatic and renal affections in dogs with special reference to Leptospirosis

Name of the Student : **Rachana Manurkar**

Name of the Advisor : Dr Nidhi Shrivastava

Degree/Year : MVSc/2024

Name of the University : College of Veterinary Science & AH, Mhow, Nanaji Deshmukh Veterinary Science University, Jabalpur, MP

The aim of this study was to evaluate the prevalence of hepatic and renal affections in dogs by examining hematological and biochemical changes in blood serum, as well as through urinalysis over a six month period from July to December 2023.

Leptospirosis prevalence was assessed by detecting Leptospira in urine samples and impression smears of the liver and kidney using Fontana's staining. The overall prevalence of hepatic and renal affections was found to be 6.92%. Regarding hepatic affections, the prevalence was 3.92%. Hematology revealed normocytic normochromic anemia in dogs with lower Hb, PCV, TEC, thrombocytopenia, neutrophilia and leukocytosis. Left and right shifts were observed in different hepatic affections. Serum biochemistry revealed elevated levels of ALT, AST, ALP, GGT and total bilirubin. On the basis of haematobiochemical observations, various types of hepatic affections recorded in present study were 82 cases of chronic active hepatitis, 7 cases of ascites, 4 cases of jaundice and 25 cases were of unknown and mixed conditions.

The prevalence of renal affections was 2.99%. Haematobiochemical examination revealed microcytic hypochromic anemia with lower Hb, PCV and TEC. Elevated levels of serum creatinine and BUN were found. Various renal affections recorded were chronic renal failure, acute renal failure, pyelonephritis, urolithiasis. However, Leptospira could not be demonstrated in the impression smears and urine smears. The Leptospira rapicard test was utilized for serological testing of unvaccinated stray dogs, revealing that two serum samples tested positive for IgG antibodies, indicating exposure of leptospira.

Title of Thesis : Pathological studies on canine cutaneous tumours with special reference to mastocytomas

Name of the Student : **Sangamoni Navaneeth**

Name of the Advisor : Dr Supriya Shukla

Degree/Year : MVSc/2024

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The present research was conducted to study the incidence of different canine cutaneous tumours in and around Mhow and Indore region and categorize them by haematological parameters, cytology, gross appearance and histopathology. Furthermore, immunohistochemical studies of the mast cell tumours were also conducted using relevant tumour markers.

In the present study, out of 36 cases, 55.56% were benign and 44.44% were malignant tumours. The age group with the highest incidence (52.77%) was >6 to 9 years, while the lowest incidence (2.77%) was observed in the 0 to 3 years age group. Labrador Retrievers had the highest breed predisposition (36.11%) and male dogs (58.33%) were more commonly affected than females (41.66%).

Haematological analysis revealed significant findings in the malignant group, particularly for dogs aged 6 to 9 years. Compared to the benign and control groups, the malignant group exhibited lower mean values of Hb, PCV and TEC. Additionally, this group showed a significant increase in mean TLC and neutrophil levels. Conversely, within the same age group, the malignant group had significantly lower mean lymphocyte values compared to the control group.

Based on their histogenic type, 20 different cutaneous tumours were identified among the 36 cases examined. Mast cell tumours were the most common, with an incidence of 13.88%. This was followed by fibroma, fibrosarcoma and hepatoid gland adenoma, each with an incidence of 8.33%.

In toluidine blue staining, the intensity of staining and the granularcytoplasmic appearance decreased as the tumour grade increased.

AgNOR staining showed a clear correlation between the mean AgNOR count per nucleus and the histological grade of MCTs, increasing with the tumour grade. The immunohistochemical staining for Ki67 and PCNA showed a correlation between their nuclear expression levels and the tumour grade. Collectively, these histological and immunohistochemical studies play an important role in the accurate grading and assessment of these tumours, which is crucial for guiding appropriate treatment strategies.

Title of Thesis	:	Etiopathology of mortality in goats with special reference to enteric diseases
Name of the Student	:	**Neha Shukla**
Name of the Advisor	:	Dr R.C. Ghosh
Degree/Year	:	PhD/2025
Name of the University	:	College of Veterinary Science and AH, DSVC Kamdhenu Vishwavidyalya, Anjora, Durg, Chhattisgarh

The goat, commonly known as the poor man's cow, has served as a good companion to humans from ancient times. They've been domesticated for milk, meat and skin. Though they have good adaptability and are quite resistant to environmental conditions still they suffer from many diseases like pneumonia, diarrhoea, tympany, gastrointestinal parasitism etc., that negatively affect their production resulting in massive economic losses to goat keepers. Considering the fact, the present study was undertaken to study the mortality pattern, spectrum of enteric pathogens and associated pathomorphological changes to enteric diseases in goats of Durg, Rajnandgaon, Raipur and Balod districts of Chhattisgarh.

Out of 274 screened, 114 dead goats with prominent enteric lesion during necropsy procedure were included in the present work. Swabs were collected from the intestine and mesenteric lymph nodes for bacterial culture. Intestinal contents were collected for parasitological examination. Tissue samples from enteric system were used for molecular study of bacterial and viral pathogens. Representative tissue samples were used for histopathological, ultrastructural and immunohistochemical study.

The mortality rate of enteric diseases in study area was recorded as 41.6% with highest rate in 0-6 month age groups (20.8%). The female goats were found more susceptible. Moreover, the data revealed higher mortality rate in summer season. A total of 48 bacterial isolates (39 single and 9 mixed) were reported and confirmed as *Escherichia coli* (n = 37), *Salmonella enterica* (n = 9) and *Proteus mirabilis* (n = 2). The biofilm forming assay by Congo red agar method revealed 83.78% isolates of *Escherichia coli*, 33.33% strains of *Salmonella enterica* and both isolates of *Proteus mirabilis* as biofilm producer. Further, the identity of bacterial isolates was confirmed by MALDI TOF MS biotyping, PCR and 16s rRNA sequencing.

The antibiogram of *Escherichia coli* isolates displayed antimicrobial resistance to tetracycline (78.37%), ampicillin (72.97%), amoxiclav (48.64%), co-trimoxazole (45.94%), cefotaxime (40.54%), ciprofloxacin (32.43%), cefixime (24.32%), gentamicin (21.62%), norfloxacin and azithromycin (16.21%), streptomycin (13.51%) and chloramphenicol (08.10%). *Salmonella enterica* isolates, had complete (100%) resistance to gentamicin, ampicillin, streptomycin and amikacin while 66.66% to tetracycline and cefotaxime, 55.55% to amoxiclav, 33.33% to norfloxacin, 22.22% to cotrimoxazole and cefixime as well as 11.11% to chloramphenicol. Both the isolates of *Proteus mirabilis* showed resistance to amikacin, ciprofloxacin, gentamicin, chloramphenicol and co-trimoxazole.

In present study, 11 cases were confirmed for PPR virus infection, based on RT PCR and nucleotide sequencing targeting N gene. Moreover, among parasites *Haemonchus* spp., *Oesophagostomum* spp., *Strongyloides* spp., *Trichostrongylus* spp., *Trichuris* spp., *Moniezia* spp. and *Eimeria* spp. were identified as individual or mixed infection based on morphological and morphometric characterization of eggs and third stage larva. Nine species of *Eimeria* were identified as *E. arloingi*, *E. ninakohlyakimovae*, *Eimeria alijevi*, *E. hirci*, *E. caprovina*, *E. caprina*, *E. christenseni*, *E. jolchijevi* and *E. apsheronica* based on morphological and morphometric features of sporulated oocyst.

Pathomorphological alterations in intestine exhibited mainly catarrhal, haemorrhagic and necrotic enteritis. Major gross findings were observed as characteristic zebra stripes of congestion in colonic mucosa and haemorrhagic mesenteric lymph node in PPR cases, nodules on the serosa of caecum and colon (Pimply gut) due to *Oesophagostomum* spp., presence of scattered small whitish non-pedunculated nodules in intestine in *Eimeria* spp. infection, multiple necrotic foci on the surface of liver in *Salmonella enterica* infection. Chief histopathological lesions were characterized by severe depletion of lymphocytes from the cortex and medulla of mesenteric lymph node and severe congestion and haemorrhage in intestine throughout the length in case of PPR, degenerations and necrotic changes infiltrating polymorphonuclear cells in liver and lamina propria of intestine, elongated and haemorrhagic crypts, sloughing of villi and complete desquamation of superficial epithelium from mucosa of intestine, presence of tapeworm in lumen of intestine with necrotic debris, proliferative enteritis in case of *Eimeria* spp. infection and presence of parasitic foci in liver.

Ultrastructural changes were characterized by fusion, atrophy and villous loss in jejunal mucosa accompanied with enlarged crypt orifices. The necrotic and fibrinous layer completely covered the mucosa of intestine.

Immunohistochemical analysis revealed expression of VEGF cytokine in cells of intestinal tissues and mesenteric lymph node, indicating inflammatory reaction.

To conclude, the spectra of enteric pathogen in goats includes several bacteria, virus and parasites, which are silent threat to public health may lead to terrible consequences, if not addressed.

Proceedings of the South Zonal IAVP Conference - 2025

The South Zonal IAVP Conference - 2025 and the National symposium on "Synergising Academia, Industry and Clinical Practice: The Role of Pathology" was organized by the Department of Veterinary Pathology, Rajiv Gandhi Institute of Veterinary Education and Research on 12th & 13th September, 2025 at Puducherry, India.

The conference was inaugurated by Shri. Yasin M. Choudhary, IAS, Secretary (Animal Husbandry), Govt. of Puducherry. Prof. C. Balachandran, Former Vice Chancellor of TANUVAS, Chennai, Dr K. Murugavel, Dean, RIVER, Dr Manjunatha S.S., South Zonal Secretary were the guests of honor. The chief guest reiterated that the synergy between academicians, veterinarians and industry researchers will certainly be helpful in generating beneficial recommendations for the scientific and farming communities, especially in formulating the future strategies for the control of diseases of Livestock, Poultry and Wildlife. A total of 150 delegates including faculty, scientists, veterinarians and postgraduate students from various institutions and contract research organization participated.

Prof. C. Balachandran presented the keynote address on "Synergising Academia, Industry and Clinical Practice: Role of Veterinary Pathology" highlighting the evolving scope and future of veterinary pathology and emphasized the need for innovation and interdisciplinary collaboration to advance the field.

The South Zone conference consisted of six technical sessions, each featuring invited lead speakers. Dr N. Pazhanivel, Professor, TANUVAS, Chennai delivered an insightful and informative lecture on "Recent Advances in the Diagnosis of Small Animal Neoplasms" in Session I. The session included 21 oral presentations and 13 poster presentations from faculty and students.

Session II was on Pathology of Infectious Diseases of Animals, Dr N.V. Kurkure, Director of Research, MAFSU, Nagpur, Maharashtra delivered an engaging and informative lecture on "Lumpy Skin Disease: An Emerging Threat to Cattle Health" emphasizing the disease's impact, epidemiology and control strategies. This session featured 21 oral presentations from faculty and students and 10 poster presentations from students.

Session III was on Toxicopathology/Non-Infectious diseases of animals and Dr V. Sthevaan, Head, Department of Pathology, Palamur Biosciences Pvt. Ltd., Telangana presented an insightful talk on "Pathological Insights into Nonclinical Safety Evaluation of Cardiovascular and Orthopedic Implants in Non-Rodent Models" highlighting the importance of toxicopathological assessments in preclinical studies. The session included 11 oral presentations from students.

Session IV was on Clinical Pathology and Application of Artificial Intelligence in Pathology and Dr Renu Ethirajan, Chief Medical Evangelist, Sig Tuple Technologies, Bengaluru, Karnataka delivered an engaging lecture on "Artificial Intelligence in Veterinary Pathology: Emerging Applications and Future Prospects" discussing innovative AI tools and their potential to transform diagnostic pathology. This session featured 15 oral presentations from faculty and students.

Session V was on Pathology of Poultry, Pet and Aviary Birds and Dr P. Srinivasan, Professor & Head, Department of Pathology, VCRI, Namakkal gave an informative presentation on "Poultry Pathology: Diagnostic Approaches for Disease Identification" focusing on practical diagnostic techniques and recent advances in poultry disease management. This session included 12 oral presentations from faculty and students and 6 poster presentations from students.

Session VI was on Forensic/Wild Animal/Laboratory Animal Pathology and Dr Jacob Alexander, Joint Director, Directorate of Animal Husbandry, Kerala delivered an insightful presentation on the "Current Scenario of Wildlife Diseases in India" highlighting recent trends, challenges and strategies for wildlife health management. This session featured 11 oral presentations from faculty and students and 10 poster presentations from students.

The Valedictory function of the South Zonal IAVP Conference - 2025 and National Symposium was held on 13th September, 2025 in the presence of distinguished guests, faculty members and participants. The session was graced by Dr C. Balachandran, Former Vice Chancellor, TANUVAS and Dr G.A. Balasubramaniam, General Secretary, IAVP, who delivered the valedictory address highlighting the importance of collaborative research and the contribution of pathology in bridging academia, industry and clinical practice. Best presentations from each session were awarded, separately for faculty and students.

The conference proved to be an inspiring and impactful gathering, bringing together academicians, practitioners, researchers and students to share advancements, exchange of ideas and chart the future of veterinary pathology with renewed enthusiasm. The two days event successfully fostered collaboration between academia, industry and clinical practice, while serving as a vibrant platform to celebrate innovation, knowledge exchange and professional growth.

SUPERANNUATION

Prof. Gulshan Narang

Prof. Gulshan Narang, Dean, College of Veterinary Sciences, LUVAS, Hisar had approximately 33 years of professional experience and has started his carreer as Scientist, FERRO-USDA project NAHL, Ggn, INDO-VAX, Hisar (1991-1994), Assistant Professor & equivalent at CCS HAU/KVK, Kaithal (1994-2004), Associate Professor (DIO) at CCS HAU/LUVAS (2004-2010), Professor (Sr. DIO) (2010-2017), Professor & Head Veterinary Pathology (2017-2022), Co-ordinator Research (P&M) (2021-2022), Registrar (Additional Charge) (2022-2023) and Dean, College of Veterinary Sciences (2022-till date of superannuation) at LUVAS, HISAR.

Prof. Narang has been credited with Major (Mrs.) Malika Trivedi IAAVR Award in 2024 by Indian Association for Advancement of Veterinary research in recognition of best contribution to Veterinary Science and Education for their advancement in the country.

He received patent for ***"A PROCESS OF TESTING UREA IN MILK"*** from Indian Patent Office, application no. 522/DEL/2004/dt. 5.5.04 with patent no. 250500 dated 6.1.12. Developed a process for detection of Malathion pesticide in feed samples and will be applying for its patent Developed a recombinant vaccine for Infectious Bursal Disease of Poultry (2024-25) in collaboration with NRCE in 2024 during research project by my PhD student and will be applying for its patent. Prof. Narang had carried out various research projects of state schemes and externally funded projects (RKVY, ICAR-NIVEDI) as PI and Co-PI.

During his tenure he acted as Chairman, Board of Studies, Member, Board of Studies, Member, Board of Management, Member, Academic Council Member Secretary, RIC, Member Secretary, IPR cell, Nodal Officer, RTI Cell, In-charge, Wild Life Unit, LUVAS and Vice President, Veterinary College Teachers Association, LUVAS, Hisar and many more.

Prof. Narang is Life Member of various professional societies *viz.* Indian Association of Veterinary Pathology, Society for Immunology and Immunopathology, Indian Association of Microbiologist, Indian Veterinary Association, Member, Haryana Veterinary Council and Veterinary Council of India etc.

He has successfully discharged his professional duties and superannuated from LUVAS, Hisar services on 31.10.2025. The Indian Association of Veterinary Pathologists wish him a healthy, happy and peacefull family life.

OBITUARY

Dr M. Jeevana Latha Passes Away

Dr M. Jeevana Latha born on 30th November, 1978. She did her BVSc & AH at CVSc, Rajendranagar, MVSc, CVSc, Tirupati and PhD from CVSc, Rajendranagar, Hyderabad. She started her career as an Assistant Professor at the Animal Husbandry Polytechnic, Mahaboobnagar and served in different positions at constituent colleges of P.V. Narsimha Rao Telangana Veterinary University (PVNRTVU). She was elevated to Professor and University Head, Department of Veterinary Pathology, at the College of Veterinary Science, Rajendranagar, Hyderabad because of her sincere and dedicated work.

Dr Jeevana Latha guided 15 MVSc and one PhD student. She published 55 research articles and 48 popular articles. She received 12 awards between 2019 and 2025, presented papers in conferences. She was life member of 9 professional bodies and also authored book chapter. She was actively involved in teaching, diagnostic and departmental activities. She was known for her simple nature, discipline and helping attitude and was respected by colleagues and students.

She passed away on 19th October, 2025. Her sudden demise is a great loss and IAVP family extends their condolences to the departed soul.

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