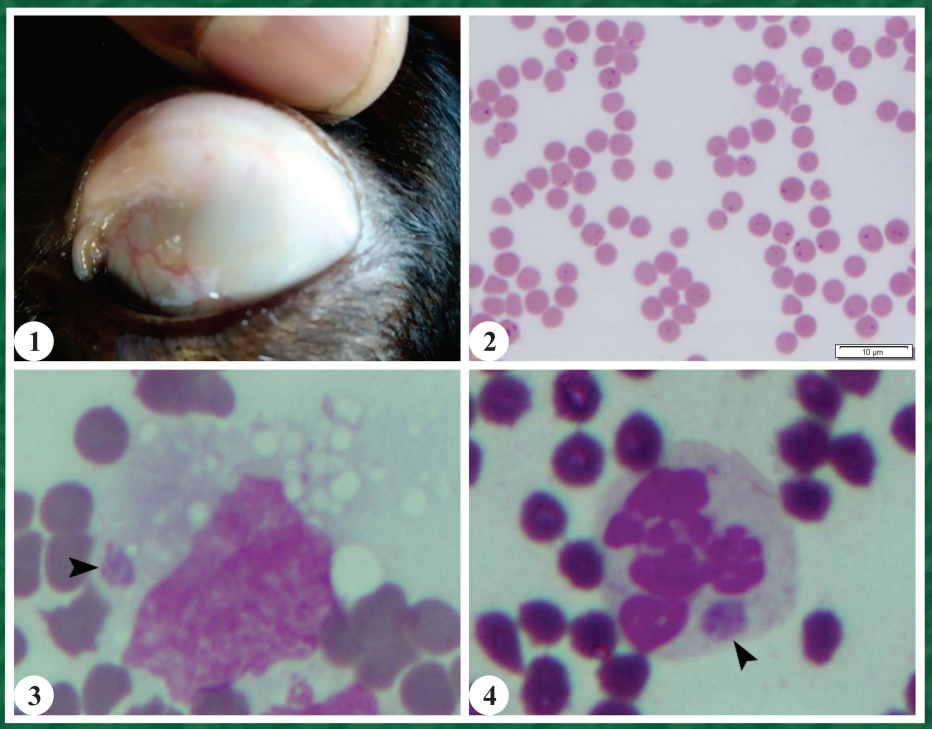
IJVP-2024

Vol.: 48(1)
March, 2024
ISSN: 0250-4758
Online ISSN: 0973-970X

INDIAN JOURNAL OF VETERINARY PATHOLOGY



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Vol. 48 (1)
March, 2024
ISSN: 0250-4758

INDIAN JOURNAL OF VETERINARY PATHOLOGY

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Indian J. Vet. Pathol., 48(1) : 1-5, 2024: DOI: 10.5958/0973-970X.2024.00001.4

RESEARCH ARTICLE

Foot-and-mouth disease in mithun, yak, cattle-yak hybrids and cattle in the north-eastern states of India during 2021-2022

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Received: 26.8.2023; Accepted: 7.9.2023

ABSTRACT

During 2021, relatively higher numbers of foot and mouth disease (FMD) outbreaks were reported in the bovine population compared to preceding years along with reports in mithun and cattle in few states of north-eastern regions of India. FMD virus (FMDV) serotype was identified using sandwich ELISA and RT-multiplex PCR on clinical samples from cattle (n = 5) and mithun (n = 11) of Arunachal Pradesh and Nagaland. The tests revealed that 2 samples from mithun and 1 sample from cattle were found positive for FMDV serotype O. Two samples from suspected FMD in mithun from an organized farm in Nagaland were tested positive for serotype A. Serum samples collected at random and tested using 3AB3 nonstructural protein ELISA revealed a higher FMDV NSP antibody prevalence in cattle-yak hybrids (16.7%), followed by mithun (10.6%) and yak (3.8%). Mithun and yak being semi-domesticated species mingle with cattle and such close proximity in common habitats and grazing areas might have caused the spread of infection. Furthermore, because of their habitat in forests, yak and mithuns are generally not vaccinated against FMD. The disease in cattle, buffalo and semi-domesticated populations of mithun and yak as well as their hybrids in hilly north-eastern parts of India is of high concern. These species need to be vaccinated biannually and monitored for protective antibody status against FMD.

Keywords: Cattle-yak hybrid, FMD, mithun, NSP ELISA, RT-mPCR, Sandwich ELISA, yak

INTRODUCTION

Mithun (*Bos frontalis*), a rare bovine species inhabiting the north-eastern hilly region (NEHR) of India, immensely contributes to the local tribal economy holding an important position in the social, cultural and religious life of the local community[1]. Being a vulnerable species, International Union for Conservation of Nature duly considers the conservation of mithun as immensely essential for India having a diverse climatic situations[2]. Foot and mouth disease (FMD), endemic in India, is caused by FMD virus (FMDV) serotypes O, A and Asia 1. It is one of the significant constraints in the way of mithun and yak farmers in the NEHR and is reported as one of the main reasons for the decline in the mithun population of Nagaland and Manipur[3]. FMD is reported as a menacing disease in these species with considerable mortality in young animals[4]. Moreover, frequent FMD outbreaks in mithun and yak have been reported during previous instances[5,6]. To cite a few, during 2009, two outbreaks due to serotype O occurred in Mizoram, causing high mortality in mithun population. Many outbreaks in yaks and hybrids of yak with local cattle have also been reported in different parts of Nepal[7,8].

The global mithun population is estimated at 0.5 million heads[9]. India has the largest mithun population (nearly 97.57%) in the world[10]. According to the 20th Livestock Census by the Department of Animal Husbandry and Dairying, the total population of mithun in the country during 2019 was 390 thousand heads and total yak was 58 thousand heads. The highest population of mithun is found in Arunachal Pradesh state constituting around 90% of the total mithun population, followed by Nagaland, Manipur and Mizoram[3]. Despite being the main support of the tribal economy of the NE region, mithuns and yaks have generally been ignored with respect to disease control measures that has predisposed them to many infectious diseases[5]. The present study reports FMD caused by serotypes O and A along with the demonstration of infection-specific antibodies in mithun, yak and cattle-yak hybrids in the north-eastern region of India during 2021-2022 that can highlight the importance of prevention of FMD in these

How to cite this article : Rout, M., Garam, G.B., Lama, R., Deka, P., Tripathy, J.P., Giri, P., Acharya, R., Subramaniam, S., Mohapatra, J.K. and Singh, R.P. 2024. Foot-and-mouth disease in mithun, yak, cattle-yak hybrids and cattle in the north-eastern states of India during 2021-2022. Indian J. Vet. Pathol., 48(1) : 1-5.

species.

MATERIALS AND METHODS

Sampling study region

The clinical samples (tongue epithelium and foot epithelium) from 11 mithuns and 5 cattle from Papum Pare district of Arunachal Pradesh and 2 clinical samples from mithun from organized herd at Nagaland were collected in virus transport medium containing 50% phosphate buffered saline and glycerol (pH 7.5). A total of 330 sera from mithun, 261 from yak and 30 from cattle-yak hybrids from West Kameng district of Arunachal Pradesh were collected during the period.

Identification of FMDV serotype in the clinical samples

Initially, the clinical samples were processed recovering the clarified homogenate further subjected to a frontline test, an antigen detection sandwich ELISA for serotype identification as described earlier[11]. Samples found negative in serotyping ELISA were subjected to a backup test, a serotype differentiating reverse transcription-multiplex polymerase chain reaction (RT-mPCR) as described previously[12]. The total RNA was extracted from the sample supernantants using the RNeasy Mini Kit (Qiagen, Germany). Reverse transcription was performed using M-MLV reverse transcriptase (Promega, USA) and reverse primer NK61[13] followed by mPCR using three serotype-specific positive sense primers namely DHP13, DHP15 and DHP9 for serotype O, A and Asia 1, respectively with the reverse primer NK61 using Hotstar Taq DNA polymerase (Qiagen, Germany). The PCR amplicons were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide and captured through gel documentation system.

Detection of FMDV nonstructural protein (NSP) antibodies

An indirect ELISA was performed to detect antibodies against FMDV 3AB3 NSP as described earlier[14]. Test sera including the negative and positive controls were diluted in diluent buffer @ 1:20, and the anti-bovine horseradish peroxidase-conjugated antibodies were used at 1:12000 dilution. Serum samples producing corrected optical density values ≥40% of those of the positive control were regarded as positive.

RESULTS AND DISCUSSION

During the year 2021, a number of suspected cases of FMD were reported in the cattle population which were confirmed in the containment laboratory at ICAR-NIFMD, Bhubaneswar[15,16]. Also, several outbreaks were reported in different districts of Arunachal Pradesh and an organized farm of Nagaland. Mithun and cattle from Arunachal Pradesh were reported with suspected FMD

Fig. 1. Foamy and ropy salivation in FMD-affected mithun; **Fig. 2.** Nasal erosive lesions in mithun affected with FMD; **Fig. 3.** Erosive lesion in palate region with foamy saliva on tongue in the oral cavity of the affected mithun.

incidences and subsequently got laboratory confirmed. The affected animals under investigation exhibited clinical signs of fever, inability to take feed, smacking of the lips with foamy and ropy salivation (Fig. 1) with lameness. Gross erosive lesions in the nasal region (Fig. 2), erosive lesion in palate region with foamy saliva on tongue in the oral cavity (Fig. 3), initial erosive lesions on the muzzle/planum nasolabiale with salivation in the affected mithun (Fig. 4), severe erosive lesions on the muzzle and nasal region with secondary maggot infestation (Fig. 5), erosions on tongue (Fig. 6) and that in the interdigital space and around the hoof suggestive of FMD were observed. Out of 11 clinical samples from mithuns and 5 cattle from Arunachal Pradesh tested in serotyping sandwich ELISA and RT-multiplex PCR, 2 samples from mithun and 1 from cattle were found positive for FMDV serotype O (Fig. 7). Before the present outbreak, FMD incidences in mithun caused by serotype O were reported during 2010 and 2011 in Arunachal Pradesh. Two samples of mithun from the organized herd from Nagaland state were found to be positive for serotype A (Fig. 7). This present study of FMD outbreak in mithun reminds an FMD outbreak long before due to serotype A in Sikkimese yak during 1973[17]. Along with the clinical samples from the affected animals, a total of

Fig. 4. Initial erosive lesions on the muzzle/planum nasolabiale with salivation in the affected mithun.

35 (10.6%) of 330 sera from mithun, 10 (3.83%) of 261 from yak and 5 (16.67%) of 30 from cattle-yak hybrids were found positive for 3AB3 NSP antibody in the indirect ELISA.

During the year 2021, relatively higher numbers of FMD outbreaks were reported in the bovine population in different states in the country compared to preceding years. Mithun and yak being semi-domesticated species, come in contact easily with cattle and the cattle-yak hybrids along with the small ruminants especially goats reared in north-eastern part of the country. Such close proximity and interactions of different susceptible animals in common habitats and grazing areas, even during shelter in the same house or shed might have triggered the spread of infection in these species. Apart from mithun, FMD (due to serotype O) has also been reported in yaks of Himachal Pradesh[18], where the animals were in frequent contact with cattle, sheep and goats being the residents of the districts bordering Tibet. In forest areas with frequent habitual nose-to-nose contact between the infected mithuns and yaks, the disease spreads to a greater number of animals. The contact of mithun and yak with migratory cattle has been predicted to have an important role in spreading the disease to these semi-domesticated animals as reported before[6]. Furthermore, because of their habitats in difficult and unapproachable forest areas, diseased yaks and mithuns are neither usually attended by the veterinarians nor the healthy animals are vaccinated against FMD thereby collectively making the animals more prone to the disease.

The Government of India is on move with the practice of routine FMD vaccination campaign in cattle and buffalo population under the livestock health and disease control programme (LHDCP) in all the states and UTs of the country, while other species have not yet been targeted for vaccination. Spillage of virus from primary hosts like cattle and buffalo to semi-domesticated populations of mithun and yak as well as their hybrids in hilly terrains of north-eastern regions is of high concern from the point of view of prevention of infectious diseases like FMD as well as the conservation of biodiversity in the region. In order to prevent the disease in these species, there is a need for regular serosurveillance and rigorous

Fig. 5. Severe erosive lesions on the muzzle and nasal region with maggot infestation in the affected mithun; **Fig. 6.** Erosions in tongue of the affected mithun.

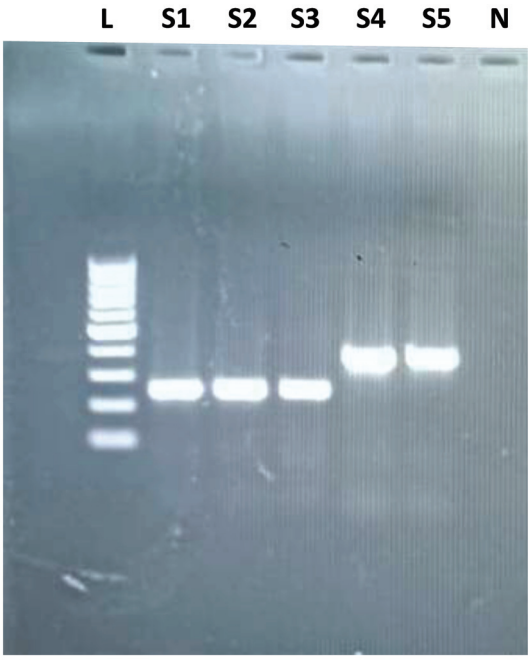

Fig. 7. RT-multiplex PCR assay showing the amplified product 249 bp and 376 bp of VP1 gene corresponding to FMDV serotype O and A, respectively analyzed by 2% agarose gel stained with ethidium bromide. Here, the positive amplification of 5 samples is shown in lanes S1, S2 and S3, S4 and S5; Lane N: Negative control; Lane L: 100 bp DNA ladder; S1, S2, S3 are samples positive for serotype O, while S4 and S5 are positive for serotype A.

vaccination strategy in the population of mithun and yak along with cattle at regular intervals that may help in breaking the virus transmission cycle. Vaccination with 100% coverage of semi-domesticated mithun and yak population at the interface of domestication is again the most vital part of FMD management[4]. Besides all these, all the stakeholders should be made aware of the clinical symptoms of FMD to promptly report the disease occurrence to the health authority along with benefits and importance of vaccination in animals for disease prevention and control.

From the present investigation and findings as well as from the earlier reports, it is clear that FMD in mithun and yak may be due to negligible herd immunity due to no/sparse vaccination, unrestricted animal movement, as well as frequent mingling of different susceptible animals in common grazing pasture land. Biosecurity practices are really very difficult to execute in habitation where mithun and yaks inhabit. Apart from this, mithun and yak being rare and unique bovines, should be given more attention on the prevention of infectious diseases like FMD with a planned vaccination strategy combined with other tools of surveillance and monitoring, as is routinely practiced for cattle and buffalo populations under national animal disease control programme in place in the country by the government of India. Continuous vigil at domestic-wildlife interface along with effective immunization in this zone is likely to be an effective strategy for FMD control in these species which are very important for preservation of biodiversity and sustenance of local livelihoods of the tribal community of the north-eastern region of the country.

ACKNOWLEDGEMENTS

We thank all those who participated in sample collection and extended technical assistance during the study. This work was done under the auspices of Indian Council of Agricultural Research.

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Indian J. Vet. Pathol., 48(1) : 6-11, 2024: DOI: 10.5958/0973-970X.2024.00002.6

RESEARCH ARTICLE

Molecular detection of *A. phagocytophilium* and *A. ovis* infection in goat flocks

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Received: 18.9.2023; Accepted: 26.10.2023

ABSTRACT

Anaplasmosis in goats is one of the most serious concerns in the fast-growing small ruminant sector, as it reduces the animal's production and weight gain. The present study was conducted to study the clinical profile, hemato-biochemical changes and molecular detection of anaplasmosis in goat flocks in and around Udgir, Maharashtra during January to December 2021. A total of 140 goats belonging to 11 rural flocks were included in present study. Among them 50 suspected cases were selected finally for screening for *A. phagocytophilium* and *A. ovis* infection by blood smear examination and PCR analysis. The blood smear examination and PCR analysis of the suspected samples (n=50), resulted 88 and 92 percent positivity for anaplasmosis, respectively. The mixed infection (*A. ovis + A. phagocytophilium)* found more prevalent in affected goats. The major clinical signs in goats recorded were fever, congested, pale or papery white mucous membranes, swollen lymph nodes, respiratory distress and lameness in few cases.The important hemato-biochemical abnormalities of anaplasmosis in goats includes marked granulocytic leukocytosis, anemia, elevated levels of serum ALT, AST, BUN, creatinine and total bilirubin. In conclusion, the goat population had 92 percent of the overall prevalence of anaplasmosis with a high incidence of mixed or coinfection (*A. ovis + A. phagocytophilium*). The clinical diagnosis of anaplasmosis in goats can be precisely made from blood smear examination but the PCR showed more specificity and sensitivity for species detection and assessment of overall prevalence. This study also reports the first molecular detection of *A. phagocytophilium* infection in Indian goat flocks.

Keywords: *Anaplasma ovis*, *Anaplasma phagocytophilium*, anaplasmosis, blood smear, goats, PCR

INTRODUCTION

Goats have been connected with man since the birth of agriculture and animal domestication. They can adapt to a wide range of environmental circumstances which makes them a very significant socio-economic animal giving goods (meat, milk, fibre, hair) and service to humans all over the world, particularly in developing nations like India[1].Vector-borne protozoal and rickettsial infections such as anaplasmosis, babesiosis and theileriosis are the serious impediment to the health of tropical livestock and cause significant economic losses due to reduced milk and meat output, mortality, abortions, expenses on treatment and control[2].

Anaplasmosis in goats is one of the most serious concerns in the fast-growing small ruminant sector, as it reduces the animal's production and weight gain[3]. Anaplasmosis causes significant losses in livestock production of countries in tropical and subtropical regions where livestock consists primarily of sheep and goats. Among various *Anaplasma* species, *A. marginale, A. centrale, A. bovis*, *A. ovis* and *A. phagocytophilium* are obligate intracellular bacteria parasitizing erythrocytes and monocytes or granulocytes of higher vertebrates, mostly ruminants[4].

In routine clinical practice, anaplasmosis is frequently diagnosed by using blood smear examination. But it is difficult to prove repeatedly the presence of inclusion bodies of *A. phagocytophilium* in leucocytes, therefore microscopic tests of blood smears should be supported by indicating the genetic material of the microbes using the PCR method[5,6].

How to cite this article : Mandhare, H.D., Chavhan, S.G., Awandkar, S.P., Jadhav, R.K., Kulkarni, M.B. and Khillare, B.S. 2024. Molecular detection of *A. phagocytophilium* and *A. ovis* infection in goat flocks. Indian J. Vet. Pathol., 48(1) : 6-11.

Although, the bovine anaplasmosis has been extensively studied in the Indian subcontinent, there is a paucity of literature on anaplasmosis occurring in Indian small ruminants, especially in caprines[3,7,8]. However, to the best of our knowledge, *A. phagocytophilium* has never been detected by molecular techniques in Indian goat population. Hence, the present study was designed to study the clinical profile, haemato-biochemical changes and molecular detection of anaplasmosis in goats.

MATERIALS AND METHODS

Selection of Animals

The present study was conducted on goat flocks in and around Udgir, Maharashtra during January to December 2021. A total of 140 goats belonging to 11 rural flocks were included in present study. Among them 50 suspected cases were selected finally for screening for *A. phagocytophilium* and *A. ovis* infection. The samples were collected randomly from goats suspected for anaplasmosis mainly comprising local Osmanabadi breed goats of all ages and both sexes with a history of fever, swollen lymph nodes, pale mucous membranes and tick infestation.

The blood smears and faecal samples from suspected goats were initially screened for presence of other parasitic infections (*viz.* theileriosis, babesiosis, coccidiosis, haemonchosis etc.). The suspected 50 goats, which were found negative in primary screening for above other parasitic diseases only were considered for further screening for anaplasmosis. The study was approved by Institutional Animal Ethics Committee (IAEC) vide resolution No. VCU/IAEC/CPCSEA/IX/21.

Collection of Blood Samples

The blood samples (n=50) from anaplasmosis suspected goats for analysing various haematological parameters, blood smear preparation and molecular studies were collected from jugular vein in EDTA vacutainers while for estimation of biochemical parameters, the blood samples were collected in clot activator vacutainers. For control purposes, the blood samples were collected from healthy goats (n=12) from Livestock Farm, College of Veterinary and Animal Sciences, Udgir of the same age, breed and sex.

Hematobiochemical Estimations

The haematological parameters such as total erythrocyte count (TEC), haemoglobin concentration (Hb), packed cell volume (PCV), total leucocyte count (TLC), absolute granulocyte, lymphocyte and monocyte count were estimated by using fully haematology analyzer (Abacus Junior Vet, Diatron GMBH, Austria). The serum biochemical parameters such as aspartate aminotransferase (AST), alanine transaminase (ALT), total bilirubin, blood urea nitrogen (BUN) and creatinine were on a semi-automated clinical chemistry analyzer (Chem-7, Erba Mannheim) by using standard commercial biochemical kits.

Preparation, Staining and Examination of Blood Smears

Thin blood smears were prepared from blood samples of suspected goats which were collected from jugular vein for haematological analysis in EDTA vacutainers. The prepared blood smears were air-dried, fixed with methanol and subjected for Giemsa staining as per routine protocol. Positive blood samples were selected on the basis of presence of intra-erythrocytic one or more small round dark basophilic inclusion bodies located centrally, sub-marginally or marginally for *A. ovis* and intra-cytoplasmic morulae made up of numerous delicate punctiform initial bodies (rod, round or oval shaped) within neutrophils or mononuclear cells of colours ranging from dark blue to purple-grey placed in vacuole surrounded by a membrane for *A. phagocytophilium*.

DNA Extraction and PCR

Total DNA was extracted from the blood samples using the DNA extraction kit (GSure Blood DNA mini-Kit, GCC Biotech, India) following the manufacturer's instructions. The DNA samples were subjected for co-amplification of partial MSP4 gene of *A. ovis* and 16S gene of *A. phagocytophilium* using F-5'-TCATTCGACATGCGTGAGTCA-3', R-5'-TTTGCTGGCGCACTCACATC-3'and F-5'-AGTGCTGAATGTGGGGATAATTTATCTCCGTG-3', R-5'-CTAATCTCCATGTCAAGGAGTGGTAAGGTTT-3' primer sets described by earlier researchers[9,10]. The PCR was conducted in a 25 µl volume at the annealing temperature of 56°C for 30 sec and the amplicons were visualized in gel documentation system.

Statistical Analysis

The data generated from different parameters of present study was subjected to independent samples t-test by using IBM SPSS software (version 20) for windows.

RESULTS

The results revealed anaplasmosis in all the goat flocks under study. Out of 50 suspected samples that were screened for anaplasmosis, forty-four (n=44, 88%) and forty-six (n=46, 92%) goats were found positive

Table 1. Prevalence of anaplasmosis in goats by blood smear examination and PCR.

Diagnostic technique	Total no. of goats tested	Total no. of goats found positive	No. of goats infected with			Total no. of goats found negative
			One pathogen		Two pathogens (mixed infection)	
			A. ovis	*A. phagocytophilium*	*A. ovis* + *A. phagocytophilium*	
Blood smear examination	50	44 (88%)	5 (10%)	3 (6%)	36 (72%)	6 (12%)
PCR	50	46 (92%)	7 (14%)	7 (14%)	32 (64%)	4 (8%)

Fig. 1. Clinical signs of anaplasmosis: **a.** Presence of tick infestation in affected goats. **b-d.** Colour of mucous membranes in affected goats:- Congested **(b)**, Pale **(c)** and Papery White **(d)**.

for anaplasmosis by blood smear examination and PCR, respectively. The blood smear examination revealed 10, 6 and 72 percent positivity for *A. ovis*, *A. phagocytophilium* and both pathogens (*A. ovis* + *A. phagocytophilium*) respectively. The data regarding the species-wise prevalence of anaplasmosis by PCR recorded an equal infection among tested animals for *A. ovis* and *A. phagocytophilium* (14%). While the mixed infection (*A. ovis* + *A. phagocytophilium*) was observed in 64 percent goats (Table 1).

The age-wise prevalence of anaplasmosis in goats was found higher in the age group above 12 months (n=32, 69.56%) followed by the age group 3 to 12 months (n=12, 26.09%) and the lowest prevalence was recorded in the age group below 3 months (n=2, 4.35%). The analysis of sex-wise data revealed higher occurrence of anaplasmosis in females (n=42, 91.30%) as compared to males (n=4, 8.70%).

The anaplasmosis positive goats showed clinical signs such as fever (103.69 ± 0.11 *vs* 100.92 ± 0.12°F), anorexia, presence of tick infestation, congested, pale or papery white mucous membranes (Fig. 1), swollen lymph nodes and respiratory distress. In few cases, clinical signs such as lameness, nasal discharge and recumbency were also recorded.

The blood smear examination of anaplasmosis suspected goats which were positive for *A. ovis* revealed presence of intra-erythrocytic one or more small round dark basophilic inclusion bodies located centrally, sub-marginally or marginally (Fig. 2), while the intra-cytoplasmic morulae made up of numerous delicate punctiform initial bodies (rod, round or oval-shaped) within neutrophils or mononuclear cells of colours ranging from dark blue to purple-grey placed in vacuole surrounded by a membrane were evident in blood smears of suspected goats found positive for *A. phagocytophilium* (Fig. 3).

The haematological analysis of blood samples of anaplasmosis positive goats (n=46) revealed a highly significant increase ($p<0.01$) in total leucocyte count and absolute granulocyte count as compared to healthy control goats (n=12). Whereas, a highly significant ($p<0.01$) decrease in total erythrocyte count, haemo-

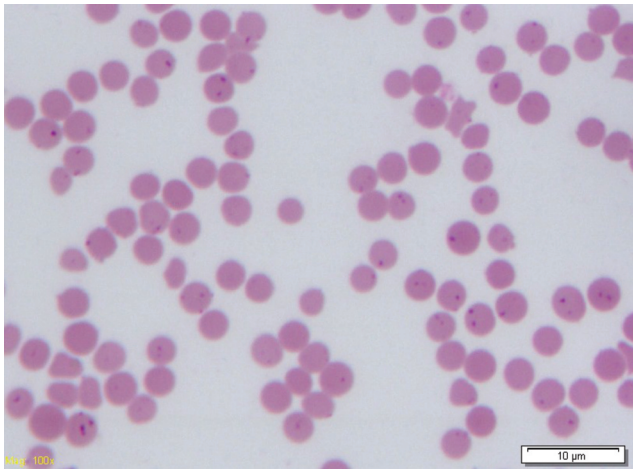

Fig. 2. Blood smear showing one or more intra-erythrocytic small round dark basophilic inclusion bodies located centrally, sub-marginally or marginally, *Anaplasma ovis* (Giemsa Stain, Bar = 10 µm).

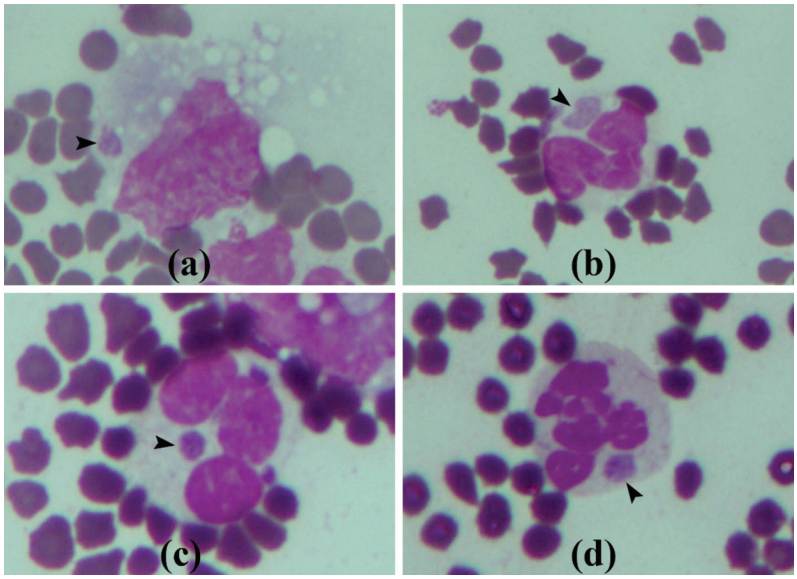

Fig. 3. Blood smear showing intra-cytoplasmic *Anaplasma phagocytophilium* morulae within **a.** Mono nuclear cell and **b-d.** Neutrophils of colours ranging from dark blue to purple-grey (arrow heads) (Giemsa Stain, Magnification = 1000X).

Table 2. Haematological changes of anaplasmosis in goats (Mean ± SE).

S. No.	Parameter	Infected (n=46)	Healthy Control (n=12)	't' value
1.	TEC (10^{12}/L)	11.38 ± 0.33	14.23 ± 0.43	4.46**
2.	Hb (g/dl)	6.13 ± 0.21	8.48 ± 0.11	6.08**
3.	PCV (%)	18.37 ± 0.57	24.89 ± 0.36	6.24**
4.	TLC (x10^9/L)	14.48 ± 0.76	11.56 ± 0.12	2.11*
5.	Granulocytes (x10^9/L)	7.34 ± 0.39	4.92 ± 0.24	3.34**
6.	Lymphocytes (x10^9/L)	7.00 ± 0.56	6.40 ± 0.28	0.58NS
7.	Monocytes (x10^9/L)	0.14 ± 0.01	0.23 ± 0.07	2.01*

NS : Non-significant, *Significant (P<0.05), **Highly Significant (P<0.01)

Table 3. Biochemical changes of anaplasmosis in goats (Mean ± SE).

S. No.	Parameter	Infected (n=46)	Healthy Control (n=12)	't' value
1.	Total Bilirubin (mg/dl)	0.39 ± 0.03	0.24 ± 0.01	2.20*
2.	SGOT (AST) (IU/L)	121.43 ± 6.59	70.65 ± 4.08	3.69**
3.	SGPT (ALT) (IU/L)	23.62 ± 0.80	18.37 ± 1.12	3.01**
4.	Creatinine (mg/dL)	0.92 ± 0.02	0.88 ± 0.03	3.72**
5.	BUN (mg/dL)	23.50 ± 1.36	12.07 ± 0.72	4.43**

*Significant (P<0.05), **Highly Significant (P<0.01)

globin concentration and packed cell volume as well as in absolute counts of monocytes were evident in anaplasmosis affected goats. The absolute lymphocyte count from anaplasmosis affected and healthy control goats not revealed any significant difference (Table 2).

Biochemical analysis of serum samples from anaplasmosis positive goats (n=46) revealed a highly significant increase (p<0.01) in serum levels of AST, ALT, creatinine and blood urea nitrogen whereas a significant increase (p<0.05) in the levels of total bilirubin were observed in anaplasmosis infected goats as compared to healthy control (Table 3).

For species detection, partial MSP4 gene of *A. ovis* and 16S rRNA gene of *A. phagocytophilium* were targeted by PCR. The PCR resulted co-amplification of 92 bp and 172 bp amplicons specific to the primer pair positions, respectively (Fig. 4). The PCR detected equal presence of *A. ovis* and *A. phagocytophilium* infection in 14 percent samples, while the mixed infection or coinfection (*A. ovis* + *A. phagocytophilium*) was recorded in 64 percent samples indicating overall prevalence of 92% in clinically suspected goats.

DISCUSSION

The clinical signs of anaplasmosis observed in goats in the present study were in agreement with the findings reported by earlier research workers[11,12]. Endogenous pyrogens generated by the *Anaplasma* organisms damage the erythrocytes and activate numerous haemopoietic and thermo regulatory centres throughout the body resulting in anaemia, high fever, weakness and weight loss[13]. The pale mucous membranes found in our study could be related to anaemia which is caused by immune-mediated erythrocyte destruction in which the host produces auto-antibodies against *Anaplasma* species and its erythrocytes. The antibodies cover parasitized erythrocytes,

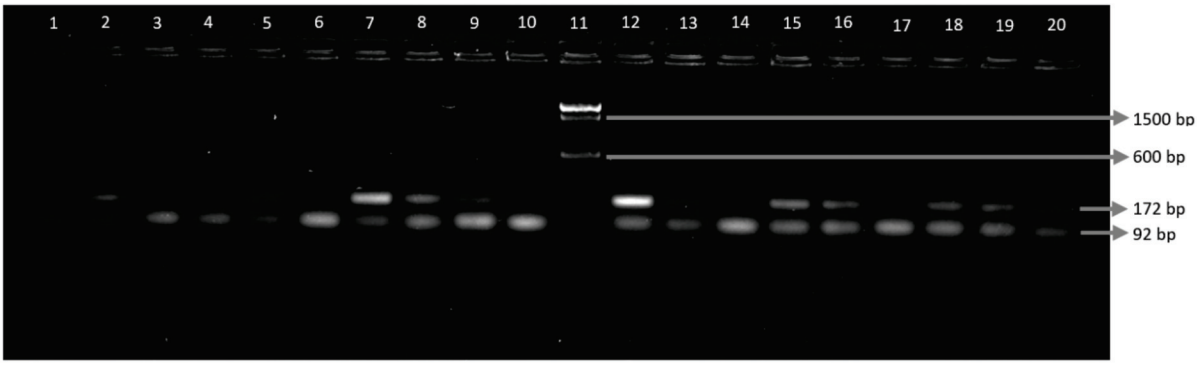

Fig. 4. Co-amplification PCR indicating specific amplification of 92 bp product for *A. ovis* and 172 bp for *A. phagocytophilium* (Lane 11: 100 bp DNA marker).

which are phagocytosed by the spleen, liver and bone marrow's mononuclear phagocytic system[14].

In the current study, highest age-specific prevalence of anaplasmosis was seen in female goats older than 12 months of age which is found consistent with findings of earlier workers[15]. Furthermore, they stated that the adult animals have more opportunities for exposure to ticks carrying the pathogen than young animals. This could be explained by the fact that the adult goats were more exposed to tick infestation carrying *Anaplasma spp.* because they went through more tick seasons.

The high prevalence of anaplasmosis in female goats can be attributed to extended breeding and lactation, stress of breeding, milking and repeated hormonal changes associated with the pregnancy and parturition[15,16]. These findings of earlier research workers were in agreement with findings of present study.

In routine clinical practice, anaplasmosis is frequently diagnosed by using blood smear examination. But it is difficult to prove repeatedly the presence of inclusion bodies of *A. phagocytophilium* in leucocytes, therefore microscopic tests of blood smears should be supported by indicating the genetic material of the microbes using the PCR method[5,6]. These findings recorded regarding diagnostic morphology of *A. ovis* and *A. phagocytophilium* in positive blood smears and findings observed during molecular detection of these pathogens in present study were also in consonance with previous reports in different animal species[6,9,10,17-20].

More or less similar haematological findings of anaplasmosis in goats were reported by earlier investigators[7,8,13,16,21,22]. Furthermore, these earlier workers stated that the significant decrease in erythrocyte count, haemoglobin concentration and packed cell volume might be due to extravascular haemolysis of erythrocytes caused by parasitic damage to erythrocytes which is phagocytosed by the reticuloendothelial system[13]. A significant increase in total leucocyte count might be due to parasite and its toxins which stimulates lymphoid tissues and stem cells in the bone marrow for phagocytosis of infected erythrocytes[21].

The biochemical changes observed in the present study were found in consonance with earlier studies of anaplasmosis in goats[21,23-25]. The increased levels of total bilirubin in anaplasmosis affected goats can be correlated with haemolysis of parasitized erythrocytes in the reticuloendothelial system, hepatic dysfunction and haemolytic anaemia whereas the increase in serum creatinine and BUN levels in anaplasmosis affected goats can be attributed to kidney dysfunction and muscle catabolism. The elevated AST and ALT level in the anaplasmosis affected animals is attributed to hepatic injury due to an increased load of phagocytosed parasitized erythrocytes in the hepatic reticuloendothelial system[24].

This study utilized MSP4 and 16S RNA genes for speciation of *Anaplasma ovis* and *A. phagocytophilium*. These genes were also used by earlier researchers for differentiation of *A. ovis* and *A. phagocytophilium* infection in goats[9,10]. As compared to the findings of present study, earlier researchers recorded more or less similar prevalence of *A. ovis* and *A. phagocytophilium* infection in goats by blood smear examination and PCR from China and other countries[26-29].

In conclusion, our study documented 92 percent overall prevalence of anaplasmosis in the goat population with a high incidence of mixed infection or coinfection (*A. ovis* + *A. phagocytophilium*). Although the clinical diagnosis of anaplasmosis in goats can be precisely made from blood smear examination, the polymerase chain reaction (PCR) showed more specificity and sensitivity in terms of species detection and assessment of overall prevalence. This study also reports the first molecular detection of *A. phagocytophilium* infection in Indian goat flock.

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Indian J. Vet. Pathol., 48(1) : 12-17, 2024: DOI: 10.5958/0973-970X.2024.00003.8

RESEARCH ARTICLE

Pathomorphology of spontaneous kidney lesions in slaughtered pigs

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Received: 26.8.2023; Accepted: 29.9.2023

ABSTRACT

The present study was conducted to know the occurrence of various spontaneous kidney lesions in slaughtered pigs and to describe their pathomorphological features. A total of 320 slaughtered pigs of either sex and mostly of marketable age were screened for the possible gross renal lesions in different regions of Krishna District in Andhra Pradesh state. Gross and microscopic examination revealed renal lesions of pathological significance in 115 animals (35.94%). Histopathologically, the lesions were categorised as vascular changes (15.65%), nephrosis (7.83%), nephritis (67.83%) and renal cysts (8.69%). Vascular changes were comprised of renal congestion (12.17%) and renal hemorrhage (3.48%). Nephritis was categorized into glomerular (30.44%), interstitial (36.52%) and tubulo-interstitial (0.87%) types. Glomerulonephritis was comprised of acute (3.48%), sub-acute (24.35%) and chronic (2.61%) types; while interstitial nephritis included sub-acute (33.04%) and chronic (3.48%) types. The present study infers a significant occurrence of spontaneous renal lesions in slaughtered pigs; the most common pathological condition being interstitial nephritis and the most consistent lesion being mononuclear cellular infiltration.

Keywords: Kidney, lesions, pigs, slaughter, spontaneous

INTRODUCTION

Pig rearing has become an important livestock enterprise in recent times because of increased awareness in the public and increased demand for pork as source of animal-based protein. Pigs are susceptible to wide array of infectious and non-infectious pathological conditions that result in significant economic losses due to mortality, loss of productivity, reduced market value and trade restrictions[1].

Renal affections in pigs constitute a significant component of organ pathologies as like in other food animals[2-4]. Spontaneous renal lesions in slaughtered pigs were well recognized and reported worldwide[5-7]. Although clinically significant disease may not be encountered, pathological conditions affecting the functionality of kidneys can result in significant production losses in terms of poor weight gain and condemnation of organs during slaughter[8-10].

Renal lesion occur either as primary conditions like cystic kidneys, nephritis, parasitic conditions and neoplasms[6,11,12] or in association with other systemic infections like swine fever, porcine circoviral infections, leptorspirosis etc.[13-16]. In this backdrop, the present study was conducted to investigate the general occurrence of various spontaneous kidney lesions in slaughtered pigs and to understand their pathomorphology in detail.

MATERIAL AND METHODS

The present study was conducted at the Department of Veterinary Pathology, NTR College of Veterinary Science, Gannavaram from December 2020 to November 2021. Pigs of either sex and mostly of marketable age that were slaughtered at various locations of Krishna District of Andhra Pradesh state and those slaughtered at the Dept. of Livestock Products Technology, NTR College of Veterinary Science, Gannavaram were included in the study. Kidneys were examined for any possible gross lesions and representative tissue samples were collected in 10 percent neutral buffered formalin. Fixed tissue samples were subjected to routine histopathological processing of paraffin embedding and microtomy sectioning. 4-5 µm thick tissue sections were then stained by standard Haematoxylin and Eosin (H&E) method for histopathological evaluation[17].

How to cite this article : Padmini, V., Hemanth, I., Devi, V.R., Rao, T.S. and Chowdary, C.S.R. 2024. Pathomorphology of spontaneous kidney lesions in slaughtered pigs. Indian J. Vet. Pathol., 48(1) : 12-17.

RESULTS

Out of 320 kidneys examined, 115 (35.94%) kidneys revealed definitive lesions of various types upon gross and histopathological examination. The various pathological conditions were

broadly categorised as shown in Table 1.

Vascular changes

In the present study, vascular changes that comprised of renal congestion (14, 12.17%) and renal hemorrhages (4, 3.48%) were recorded in 15.65% (18) cases. Grossly, kidneys with congestion were slightly enlarged and dark red in color; while renal hemorrhages appeared either as diffuse petechiae or as large ecchymotic hemorrhages. Microscopically, kidneys with congestion revealed engorged inter-tubular blood vessels and glomerular capillaries; while renal hemorrhages were characterized by extravasated RBCs in the interstitium and within the tubules along with few diffuse areas of extensive hemorrhages.

Nephrosis

Nephrosis was noticed in 7.83% (9) cases.

Table 1. Occurrence of spontaneous kidney lesions in pigs.

S.No.	Type of condition	No. of cases	Percent (N=115)
1.	Vascular changes	18	15.65
	Renal congestion	14	12.17
	Renal hemorrhages	4	3.48
2.	Nephrosis	9	7.83
3.	Nephritis	78	67.83
	Glomerulonephritis	35	30.44
	Acute glomerulonephritis	4	3.48
	Sub-acute glomerulonephritis	28	24.35
	Chronic glomerulonephritis	3	2.61
	Interstitial Nephritis	42	36.52
	Sub-acute interstitial nephritis	38	33.04
	Chronic interstitial nephritis	4	3.48
	Tubulo-interstitial nephritis	1	0.87
4.	Renal Cysts	10	8.69

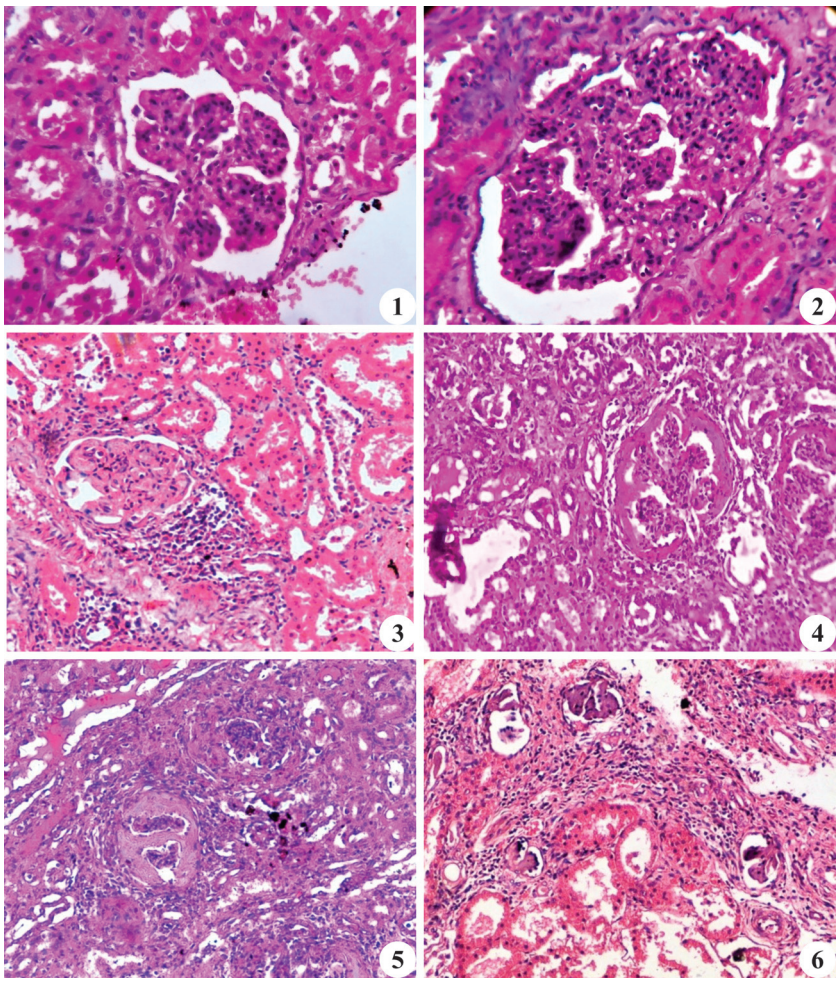

Fig. 1. Acute glomerulonephritis: Digitation of glomerular tuft with mesangial cell proliferation (H&E x400); **Fig. 2.** Acute glomerulonephritis: Synechia formation by fusion of glomerular tuft with Bowman's capsule (H&E x400); **Fig. 3.** Sub-acute glomerulonephritis: Solid & sclerotic glomerulus with peri-glomerular MNC infiltration (H&E x100); **Fig. 4.** Sub-acute glomerulonephritis: Epithelial crescent completely encircling the glomerular tuft (H&E x100); **Fig. 5.** Sub-acute glomerulonephritis: Epithelial crescent invading and completely dissecting the glomerular tuft (H&E x100); **Fig. 6.** Chronic glomerulonephritis: Severe atrophy of glomeruli leaving a small tuft of capillaries along with cellular infiltration (H&E x100).

Grossly, the kidneys appeared enlarged and pale. Microscopically, diffuse areas of tubular degeneration were noticed, characterized by cloudy swelling and vacuolar degeneration of lining epithelial cells.

Nephritis

Nephritis was observed in 67.83% (78) cases that include glomerulonephritis (35, 30.44%), interstitial nephritis (42, 36.52%) and tubulo-interstitial nephritis (1, 0.87%). Glomerulonephritis was categorized as acute, sub-acute and chronic types, and interstitial nephritis as sub-acute and chronic types.

Glomerulonephritis

Acute glomerulonephritis

It was noticed in 3.48% (4) cases. Grossly, the kidneys were enlarged, pale and edematous with multiple reddish foci on the cortex. Microscopically, congested and swollen glomeruli were noticed which revealed hypercellularity with mild mesangial cell proliferation, increased lobulation/ digitation of glomerular tufts (Fig. 1) and synechiae formation with Bowman's capsule (Fig. 2). Narrowing of Bowman's space and accumulation of proteinaceous fluid in the lumen were also noticed.

Sub-acute glomerulonephritis

It was noticed in 24.35% (28) cases. Grossly, kidneys appeared enlarged and pale with slightly irregular cut surface. Widening and yellowish discoloration of cortex was also noticed. Microscopically, diffuse glomerular lesions characterized by formation of epithelial crescents and synechiae were the predominant findings. Hypercellularity with mesangial cell and endocapillary proliferation was noticed giving a solid and sclerotic appearance to the glomeruli (Fig. 3). Epithelial crescents were composed primarily of denuded and proliferating epithelial cells which sometimes admixed with fibrin and inflammatory cells. Crescents were seen encircling the glomerular tufts either partially or completely (Fig. 4), sometimes even dissecting the glomerulus into two halves (Fig. 5). Segmental or global glomerular necrosis was also noticed at multiple areas with infiltration of neutrophils and mononuclear cells (MNCs).

Chronic glomerulonephritis

It was noticed in 2.61% (3) cases. Grossly, kidneys were smaller and hard with a finely granular surface. Capsule revealed adhesions with the cortex. Microscopically, diffuse glomerular sclerosis, shrinkage and atrophy were the predominant findings. Sclerosed glomeruli appeared hypercellular and solid with widened Bowman's space. Some glomeruli were severely atrophied leaving a small, stalk like capillary tuft (Fig. 6); while a few were completely reduced to a fibrous nodule with hyalinization and disappearance of capillaries (Fig. 7). Glomerular necrosis with eosinophilic cellular debris, denuded endothelial cells and infiltrating MNCs was also noticed. Extensive fibrous tissue proliferation with MNCs infiltration was noticed adjacent to atrophied glomeruli and degenerating tubules.

Interstitial nephritis

Sub-acute interstitial nephritis

It was noticed in 33.04% (38) cases. Grossly, the affected kidneys revealed whitish to yellowish white foci on the surface. Histologically, multiple areas of extensive MNCs infiltration into the interstitium were the significant findings. These cells were seen either as nodular aggregates (Fig. 8) or as locally extensive infiltration (Fig. 9). Periglomerular, inter-tubular and perivascular infiltration was also evident (Fig. 10). In addition, tubular degeneration and tubular necrosis along with multiple, irregularly dilated cystic tubules were also observed.

Chronic interstitial nephritis

It was noticed in 3.48% (4) cases. Grossly, the kidneys were small and hard with rough surface and prominent capsular adhesions. Microscopically, diffuse and extensive proliferation of fibrous tissue was noticed in the interstitium along with MNCs infiltration (Fig. 11). Cystic dilatation of tubules were noticed, which at some places ruptured to form giant cystic spaces within the interstitium. Glomeruli revealed sclerosis, shrinkage and hyaline degeneration. Hypertrophy of blood vessels, focal areas of necrosis and dystrophic calcification were the other findings.

Tubulo-interstitial nephritis

It was noticed in 0.87% (1) cases. Grossly, the kidney was slightly enlarged with multiple, slightly elevated, greyish white foci on the surface. Microscopically, multi-focal areas of tubular necrosis characterized by liquefaction and cellular infiltration by polymorphs and MNCs were observed (Fig. 12). Few dilated tubules containing cellular debris and inflammatory cells were also noticed. Interstitium revealed moderate infiltration of lymphocytes, plasma cells and few neutrophils along with destruction of all tubular elements.

Renal cysts

Renal cysts were noticed in 8.69% (10) cases. Grossly, cysts were noticed either solitarily or in multiple numbers. Bilateral involvement was also observed in few animals. Size of the cysts varied from 1-2 cm to 4-5 cm in diameter involving either cortex or medulla or both. Upon incision, the cysts revealed clear watery fluid with a thick, whitish limiting membrane. Microscopically, empty or fluid filled cystic cavities were noticed lined by either single layer of flattened epithelium or 2-3 layers of low cuboidal epithelium. Connective tissue proliferation was noticed beneath the lining epithelium encircling the small, thick-walled cystic tubules.

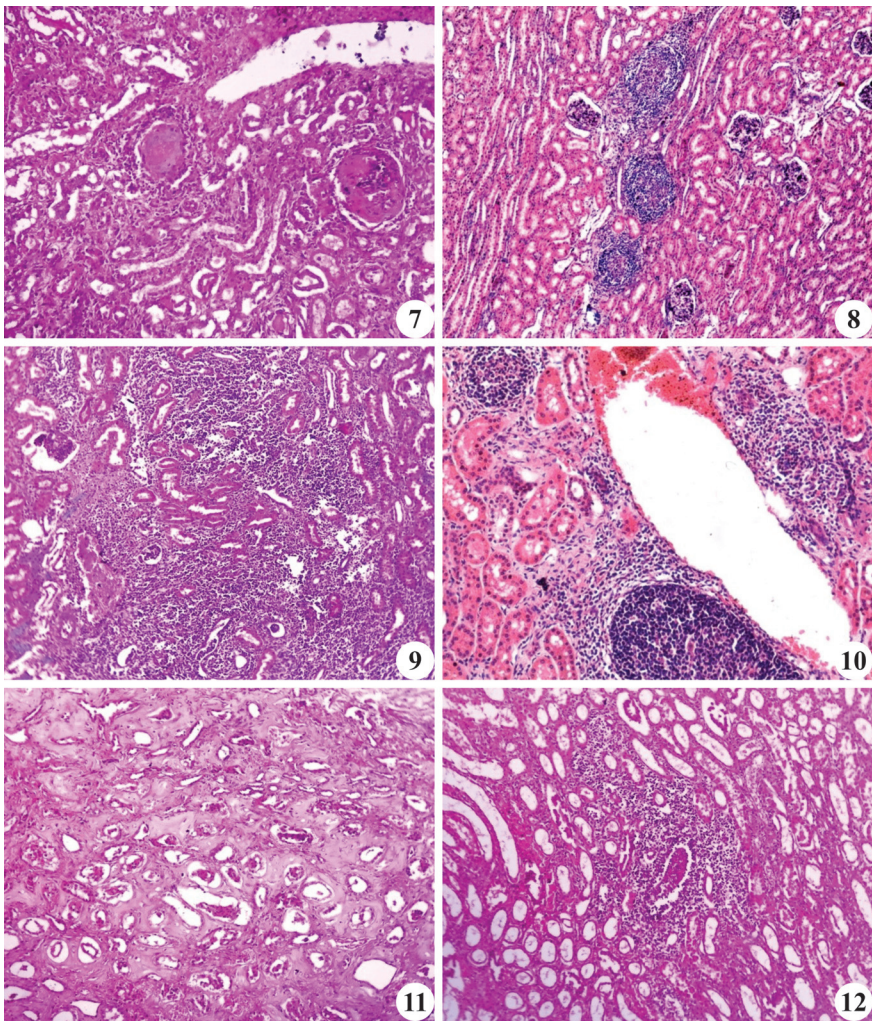

Fig. 7. Chronic glomerulonephritis: Fibrosed glomeruli with complete hyalinization of capillary tufts (H&E x100); **Fig. 8.** Sub-acute interstitial nephritis: Nodular aggregation of MNCs within the interstitium (H&E x100); **Fig. 9.** Sub-acute interstitial nephritis: Diffuse and extensive infiltration of MNCs in the interstitium (H&E x100); **Fig. 10.** Sub-acute interstitial nephritis: Peri-vascular nodular aggregation of MNCs (H&E x100); **Fig. 11.** Chronic interstitial nephritis: Extensive interstitial connective tissue proliferation with degenerating tubules lumen (H&E x100); **Fig. 12.** Tubulo-interstitial nephritis: Necrosis and liquefaction of renal tubule surrounded by infiltrating inflammatory cells (H&E x100).

DISCUSSION

Out of 320 slaughtered pigs examined, detectable gross and microscopic renal lesions were noticed in 35.94% (115) animals. A lower occurrence of 19.42%[7] and similar occurrence of 34.5%[18] were reported earlier. The variation might be due to the differences in the geographical area, duration of study undertaken and also the sample size involved.

Vascular changes comprising of renal congestion and hemorrhage were recorded in 15.65% (18) cases. Vascular changes suggest either a local pathological condition or some systemic conditions like septicemias. Renal haemorrhages are commonly associated with bacterial septicemias, acute glomerulonephritis, acute intoxication, electrocution and some acute viral diseases[19].

Nephrosis which refers to the degenerative changes of kidney is the most commonly encountered pathological condition. The gross and histological findings observed are in agreement with earlier reports[4]. Nephrosis usually results due to mild etiological factors like hypoxia and exposure to sub-lethal doses of heavy metals (mercury, lead, arsenic), toxic plants, oxalates, and antibacterial and antifungal drugs[19].

Nephritis constitutes an important category of renal diseases in various animal species. An incidence of 20% and 24% were reported in calves[20] and sheep[21] respectively. In the current investigation, nephritis was the most predominant pathological condition observed (67.83%) which was in accordance with earlier studies[8,22].

Glomerulonephritis with its different types *viz.* acute, sub-acute and chronic comprised of 30.44% (35) cases. Variety of factors like drugs, chemicals, food

allergens, endogenous antigens and infectious agents were attributed to trigger the glomerulonephritis in swine[23]. In addition, several infectious diseases like classical swine fever and African Swine Fever[24], systemic cytomegalovirus infection[25] and group A streptococcal abscesses[26] usually manifest lesions pertaining to glomerulonephritis. The major histopathological findings in the current study are in agreement with earlier reports[24,27,28]. Epithelial crescents formation in the glomeruli and Bowman's space is considered to be the hallmark of sub-acute and chronic glomerulonephritis in many species[24].

Interstitial nephritis has been reported earlier in various species like sheep, goat, calves and pigs[20,29,30]. Various bacterial, viral and fungal agents, the toxins and heavy metals in the feed are attributed to be the cause. In the current study, the incidence of 36.52% (42 cases) and corresponding gross and microscopic findings are in accordance with the earlier reports[31-33]. Many haematogenous infections of bacterial pathogens like *Leptospira* spp., *Corynebacterium* spp., *Escherichia coli*, *Staphylococcus* spp. and *Streptococcus* spp. and viral agents like PRRSV, PCV2 and PPV were incriminated to induce similar multi-focal lesions of interstitial nephritis in pigs[31,34-37]. Tubulo-interstitial nephritis was observed in 0.87% (1) cases. This condition suggests either a hematogenous bacterial infection or an ascending urogenous infection[28,38]. The gross and histopathological findings are in agreement with earlier reports[28].

Renal cysts were observed in 8.69% (10) cases. However, a lower incidence of 0.26% and 2.93%, while a marginally higher incidence of 11% was recorded in earlier studies[5-7]. Gross and microscopic findings are in agreement with earlier reports[8]. Simple renal cysts were reported to be a common incidental finding in slaughter houses and an important cause of kidney condemnation[19]. While the congenital renal cysts are inherited as an autosomal dominant trait, the acquired cysts are postulated to occur secondary to interstitial fibrosis or due to exposure to certain nephrotoxic agents in animal feeds like etoxyquin (EMQ)[28,38-40].

CONCLUSION

Thus, the present study concluded that there is a significant occurrence of spontaneous kidney lesions in slaughtered pigs with nephritis being the most frequent pathological condition and mononuclear infiltration being the most consistent lesion.

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Indian J. Vet. Pathol., 48(1) : 18-25, 2024: DOI: 10.5958/0973-970X.2024.00004.X

RESEARCH ARTICLE

Study on the mortality pattern of African Swine Fever in pigs during an outbreak in Ranchi, Jharkhand

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Received: 5.9.2023; Accepted: 26.10.2023

ABSTRACT

The present study on African Swine Fever (ASF) in Ranchi, Jharkhand demonstrates the mortality pattern in different age groups of pigs during an outbreak in 2022. The pigs were also examined for clinical signs before death and, gross and histopathology after death during months of August to September were analysed, and the mortality pattern was correlated with the age of pigs at 15 days interval. Among different age groups, the mortality in the starter (0-40 days) age group was lower (19.82%) during the initial phase of the outbreak compared to grower (41-120 days) and adult-finisher (≥121 days) which was 35.15% and 78.54% respectively on 15th day. At 45th day, the mortality reached 100% in the adult finisher group whereas it was 72.69% and 82.53% for starter and grower groups respectively. The grower and starter lot showed 93.23% and 92.17% mortality respectively by the end of the outbreak (60th day) season. A comparative analysis of the overall mortality among different breeds showed a non-significant difference ($p< 0.05$). At necropsy, the prominent gross lesions were splenomegaly, haemorrhagic hepatic and mesenteric lymph nodes, epicardial and endocardial haemorrhages, haemorrhagic gastroenteritis, interstitial pneumonia, multifocal hepatic necrosis, and congested kidneys in most of the animals, where as renal petechial haemorrhages was observed in one pig which died later during the outbreak when mortality had subsided. Histopathologically, the lesions were renal tubular necrosis, hepatic sinusoidal congestion and vacuolar degeneration, massive congestion and haemorrhages in cortical and medullary areas of spleen and lymph node, heart showed haemorrhage and congestion with pyknotic changes in the cardiomyocytes.

Keywords: African swine fever, age, breed, gross pathology, histopathology, mortality pattern

INTRODUCTION

African Swine Fever (ASF) is a highly contagious disease of both farm raised as well as wild pigs. It was first detected in Kenya in the year 1921[1] from where it spread to all Sub-Saharan African countries and later in the year 1957, reported in Portugal and other European, North and South American countries[2]. Subsequently, the disease rapidly spread to Asian countries like China, Indonesia and Myanmar by 2018. In India, the disease was first recorded from Assam[3] and Arunachal Pradesh in 2020 and the possible source of ASFV was suggested to be from China[4]. ASF is classified as a notifiable disease by the World Organization for Animal Health (OIE, 2008).

Pig farming is a significant source of livelihood in Jharkhand and plays an important role in the rural economy, especially in the tribal belt. Pig farming has gained considerable popularity in the state. Most of the tribal people rear pigs in the backyard along with other livestock and poultry. Pigs also thrive well in the agro climatic conditions of the state and its meat serves as an important source of protein in the rural Jharkhand. The total pig population of Jharkhand as of 2019 census was 12.8 lakh. In 2022, suspicious death of pigs were reported in large numbers from all over Jharkhand from the month of July onwards which was later confirmed to be due to ASF by National Institute of High Security Animal Diseases (NIHSAD), Bhopal, India. The present work was taken up to study the age and breed wise mortality pattern and pathology of ASF in pigs of Ranchi and surrounding areas.

How to cite this article : Gupta, M.K., Kumar, S., Lakra, P.P., S., Rajukumar, K., Kumar, B., Pamia, J., Kumar, R., Kumar, A., Mahtha, B.B. and Prasad, S. 2024. Study on the mortality pattern of African Swine Fever in pigs during an outbreak in Ranchi, Jharkhand. Indian J. Vet. Pathol., 48(1) : 18-25.

MATERIALS AND METHODS

The study was conducted on pig mortality recorded in the Government Pig Farm (GPF) managed by Department of Animal Husbandry, Government of Jharkhand, located in Kanke area of Ranchi district, and in the Instructional Pig Farm (IPF) of College of Veterinary Sciences and Animal Husbandry, Kanke, Ranchi. Both the farms are located at a distance of 500 metres. The GPF, Kanke and IPF, CVSc & AH,

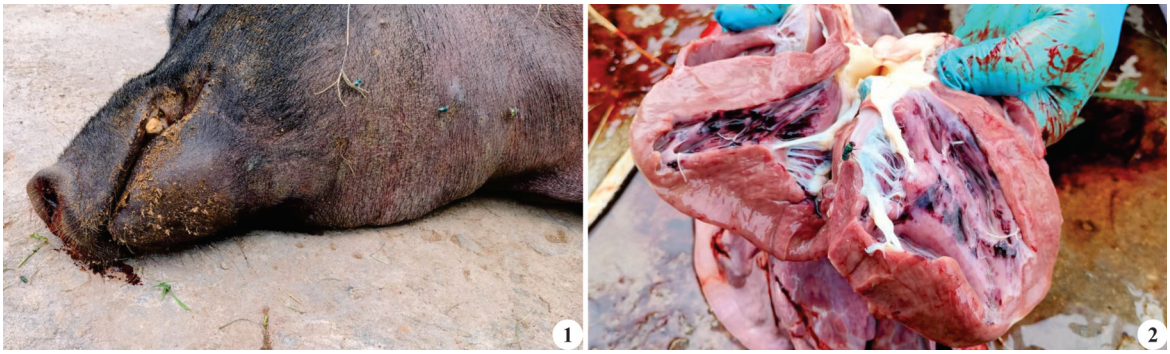

Fig. 1. ASF infected pig showing bleeding from natural orifices; **Fig. 2.** Heart showing endocardial haemorrhage on both medial ventricular walls.

Kanke, Ranchi housed 2034 and 1575 pigs of different age groups respectively at the start of the study. The following parameters were examined during the present study.

Clinical signs

The clinical signs exhibited by the animals of different age groups suffering from ASF in both the pig farms under study were critically observed and recorded from the onset of disease outbreak and during its progression.

Gross pathology

Post mortem examination was conducted on six animals in early phase of mortality in GPF and two on each day during the first three days of outbreak in IPF, Ranchi. During post-mortem examination, every organ was examined critically and the gross lesions present in different organs of dead pigs were recorded. Once the disease was confirmed, the post-mortem examination was stopped at both the places.

Histopathology

Small pieces of tissue were collected from spleen, lymph nodes, heart, stomach, intestine, lungs, liver, and kidney during necropsy and preserved in 10% buffered formalin for histopathological examination. The tissues were processed for histopathology as per the standard technique of Bancroft and Gamble[5]. The sections were stained with Haematoxylin and Eosin stain and DPX mounted permanent sections were critically examined for histopathological alterations in different organs of the body.

Confirmation of the disease

Samples of tissue from spleen, lymph nodes, heart, stomach, intestine, lungs, liver and kidney were collected during PM examination and preserved in transport media and in 10% neutral buffered formalin along with 18 whole blood samples in EDTA. The serum samples were submitted to Director ICAR-NIHSAD, Bhopal, for confirmation of the disease causing pathogen through molecular diagnostic techniques such as RT-PCR and nucleotide sequencing.

Mortality pattern

Mortality pattern in starter (0-40 days), grower (40-120 days) and adult animals (more than 120 days) of both the farms were studied at 15, 30, 45 and 60 days of the onset of clinical ASF.

Statistical analysis

The data generated during the study period was subjected to Chi-Square test as given by Snedecor and Cochran[6] to understand the statistical significance of

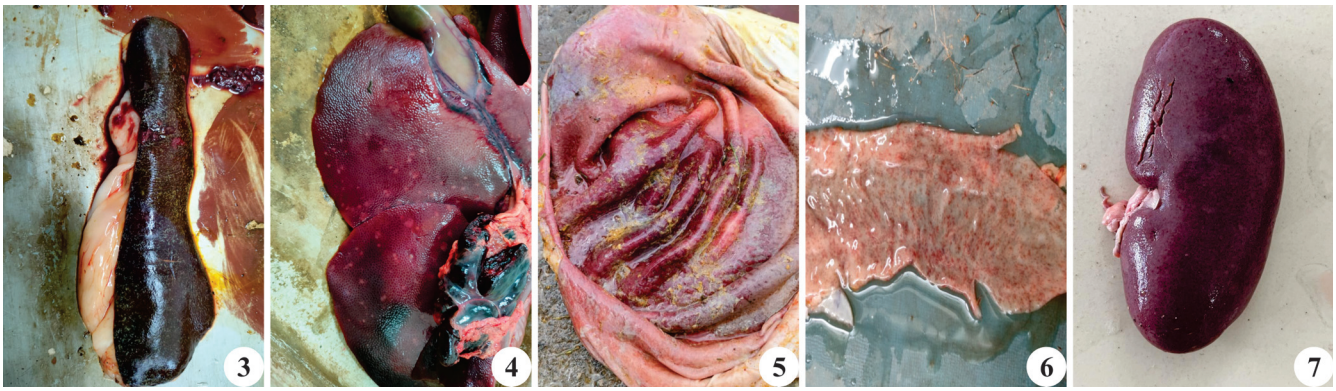

Fig. 3. Spleen showing marked enlargement and congestion; **Fig. 4.** Liver showing multifocal necrotic lesions along with severely swollen and haemorrhagic hepatic lymphnodes; **Fig. 5.** Stomach showing severe haemorrhagic gastritis with fibrinous exudates; **Fig. 6.** Intestinal mucosa showing petechial and ecchymotic haemorrhages; **Fig. 7.** Kidney showing Petechial haemorrhage (Turkey egg appearance).

variation in the mortality pattern amongst different breeds and age group of pigs.

RESULTS

It was observed that pigs reared in GPF suffered from heavy mortality at the rate of 10-15 pigs/day starting from last week of July 2022, and continued till August 2022. Overall 98.38% mortality was recorded during the outbreak, resulting into survival of 33 out of 2034 pigs. The outbreak of similar nature in the adjacent IPF started in the last week of August 2022 i.e. about one month after the initial outbreak and continued till the end of September 2022. In the extended outbreak 98.35% mortality was recorded with survival of only 20 pigs out of 1575 at the end.

Clinical signs

The diseased pigs in both the farms exhibited similar clinical signs like high fever (104-106^{O}F), depression, anorexia, bleeding from natural orifices (Fig. 1), highly congested and cyanotic skin particularly in the ventral abdomen and neck region. Death usually resulted within 24 hours of the onset of clinical signs. The disease initially affected the animals of one pen; however, later it spread to other pens. Morbidity and mortality was higher in adult animals initially, but later the disease was found to affect growers and starters too. No difference was observed in the clinical signs of the disease between different age groups and breeds of pig.

Gross Pathology

The most common organs affected during the outbreak of ASF in decreasing order of frequency were spleen, lymphnode, epicardium and endocardium of heart, lungs, stomach, small and large intestine, liver and kidney. The major consistent gross pathological lesions in the pigs of both the farms were splenomegaly, where spleen showed more than two to three times enlargement in size as compared to normal (Fig. 2). Lungs manifested bilateral pneumonia with patches of haemorrhages on its surface. Heart consistently showed marked epicardial and endocardial haemorrhage in almost all the animals died due to ASF (Fig. 3), with ventricles mostly found devoid of blood. The mesenteric and hepatic lymph nodes showed marked haemorrhagic and oedematous changes in all the animals (Fig. 4). Gastrointestinal tract revealed marked haemorrhagic gastroenteritis with development of large patch of dark red haemorrhages in the fundic mucosa of greater curvature of stomach, Intestine mainly revealed widespread petechial and linear mucosal haemorrhages all along its length (Figs. 5 and 6). None of the dead animals exhibited button ulcer in the intestinal mucosa. Liver characteristically showed multifocal hepatic necrosis with round, pale, shallow necrotic lesions measuring 3-5 mm in diameter (Fig. 4). Although both the kidneys in affected animals showed congestion, however petechiations or turkey egg appearance was not a feature in any of the affected animals except one (Fig. 7) which was recorded when death took place after acute phase of outbreak had passed. The blood examination of this animal was found to be negative for ASF.

Histopathology

Characteristic microscopic lesions were observed in visceral organ of pigs from both the farms. Spleen revealed significant increase in red pulp (Fig. 8), sub-capsular oedema and marked infiltration of mononuclear cells in the sinuses. Lymphnode showed haemorrhages in both cortical and medullary area with islands of lymphoid follicles giving characteristic washed out appearance. There was sub-capsular oedema and highly congested blood vessels (Fig. 9). Lungs revealed congestion and haemorrhage with interstitial pneumonia characterised by predominant infiltration of mononuclear cells along with few neutrophils in the interstitial spaces. Heart showed congestion and haemorrhage with pyknotic changes in the cardiocytes. Congestion and haemorrhage in submucosa and muscularis mucosa along with degeneration of glandular tissue at few places was also observed. Stomach revealed mononuclear cell infiltration in pyloric glands, highly congested large blood vessels, as well as haemorrhagic and degenerative changes in the gastric mucosa. Intestine showed haemorrhage, oedema and marked degeneration of villi (Fig. 10), as well as inflammatory cell infiltration in lamina propria

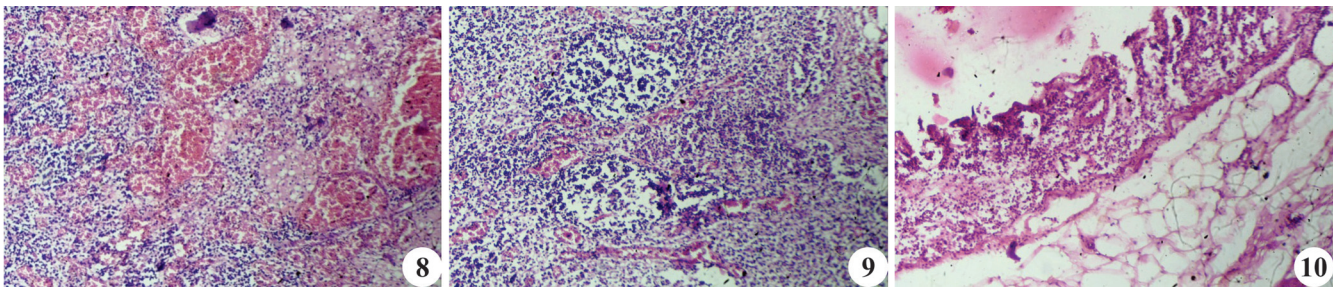

Fig. 8. Spleen showing marked increase in the red pulp due to haemorrhage (H&E x100); **Fig. 9.** Lymphnode showing washed out appearance of lymphoid follicles in ASF (H&E x100); **Fig. 10.** Intestine showing mucosal degeneration with loss of villi, and infiltration of inflammatory cells in the lamina propria and submucosa (H&E x100).

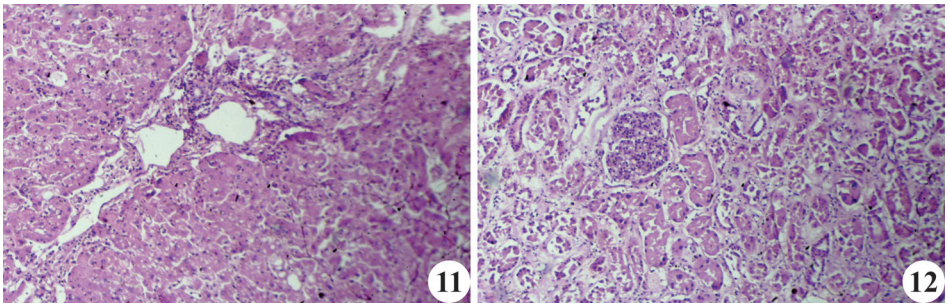

Fig. 11. Hepatocytes showing coagulative necrosis in periportal area, as well as portal hepatitis (H&E x100); **Fig. 12.** Kidney showing coagulation necrosis of tubular epithelial cells (H&E x100).

Table 1. Mortality percent in pigs of different age group at 15 days interval.

Days	Adult - Finisher	Grower	Starter
0-15	78.54%	35.15%	19.82%
16-30	95.77%	72.05%	43.47%
31-45	100%	82.53%	72.69%
46-60	100%	93.23%	92.17%

and submucosa. The inflammatory cells predominantly consisted of lymphocytes and macrophages. Liver showed highly congested sinusoids, coagulative necrosis and vacuolar degeneration of hepatocytes (Fig. 11), proliferation of bile ducts, and formation of hyaline bodies of variable size in the hepatocytes. Hepatocytes also showed pyknotic and karyorrhectic cellular changes. Moderate degree of mononuclear cell and eosinophil infiltration was observed in the portal triad. Kidney showed marked coagulative necrosis of tubular epithelium characterised by cytoplasmic acidophilia, and pyknotic nuclei (Fig. 12). At places there was complete detachment of tubular epithelium with their aggregation in the lumen. Glomerular tuft revealed hyperplastic changes.

Mortality pattern

The disease exhibited over 98% morbidity and mortality in the pigs of both the farm. The age wise and breed wise details of the mortality pattern in both the farms are presented in Figs. 13 and 14, respectively. Variable mortality percentage was observed amongst different age group of pigs irrespective of breeds. Mortality percentage in adult pig reached 78.54% (0-15 days) and 95.77% (15-30 days) by the end of first month. Moderate mortality (72.05) was observed in grower while least mortality (43.47%) was recorded in the starters during first month of outbreak. However in the second month 100% mortality was observed in adult pigs by 45th day, whereas in grower and starter pigs, mortality reached 93.2 and 92.17% by the end of 60th day (Table 1, Fig. 13). Survival was seen only in grower and starter pigs during the current outbreak. Thus, higher mortality was recorded in adult animals from the very beginning in both the farms. Chi-square analysis revealed that there was significant age wise difference in the mortality percentage in first 30 days of the outbreak (Table 2). Survival percentage of pig was found to be 1.62% and 1.65% in the GPF and IPF respectively. Breed wise variation was observed in the mortality percentage

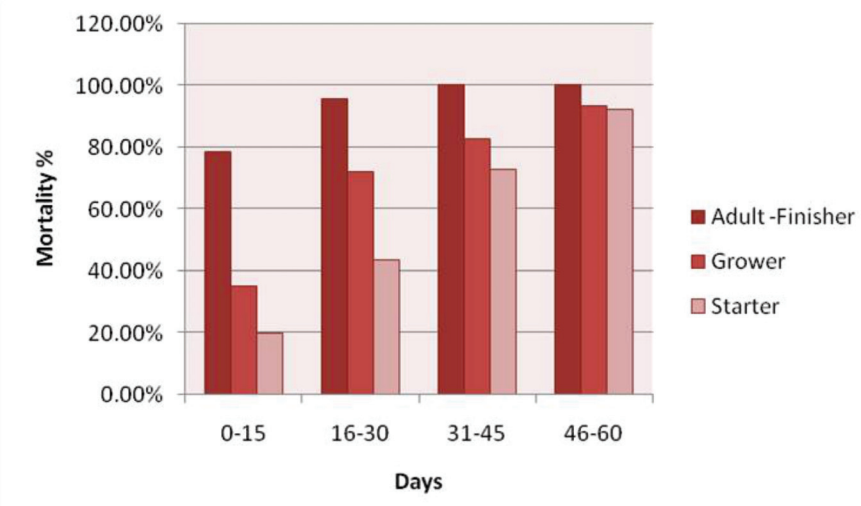

Fig. 13. Mortality trend in pigs of different age group at 15 days interval (Graph).

Table 2. Chi-Square analysis of the mortality between different age groups at 15 days interval.

Days	Between groups	P value
0-15 days	Starter x Grower	17.588**
	Starter x Adult-Finisher	115.883**
	Grower x Adult-Finisher	43.097**
16-30 days	Starter x Grower	23.449**
	Starter x Adult-Finisher	53.128**
	Grower x Adult-Finisher	7.122**
31-45 days	Starter x Grower	10.019**
	Starter x Adult-Finisher	1.798NS (p<0.05)
	Grower x Adult-Finisher	3.392NS
45-60 days	Starter x Grower	0.016NS
	Starter x Adult-Finisher	0.687NS
	Grower x Adult-Finisher	0.464NS

**Significant at p<0.01; *Significant at p<0.05; NS - Non-significant

which revealed highest mortality in Ghungroo (100%) followed by Large White Yorkshire, Jharsuk, Tamworth and Hampshire. Least mortality (69.44) was observed in Russian Charmukha breed of pig (Table 3, Fig. 14). However, the difference in mortality percentage between different breeds of pig was found to be statistically non-significant (Table 4).

Confirmation of the disease

Analysis of whole blood, serum and tissue samples was carried out at ICAR- NIHSAD, Bhopal, India by RT-PCR followed by nucleotide sequencing of the isolated virus particle isolated. Investigation of eleven samples submitted by GPF, Kanke, revealed that 4 nasal swab, 3 rectal swab, one clotted blood and three tissue samples were found positive for ASF while one sample of clotted blood was found negative for ASF. At the peak of the outbreak the positivity percentage was found to be 91.1% in whole blood, serum and tissue samples. In the subsequent outbreak at IPF, five whole blood samples, three from live and two from dead animals and five serum samples, three from live and two from dead were submitted for investigation at ICAR-NIHSAD, Bhopal, India. Test result revealed that positivity percentage in both serum and whole blood samples from dead animals was 100% while serum and whole blood samples from live animals during the peak of ASF outbreak was only 33.3%. The random samples collected after one and half month of initial outbreak from one Hampshire, one Tamworth, four Jharsuk, three LWY and one Russian Charmukha pigs of IPF were found negative for ASF. The tissue samples of pig which apparently showed classical turkey egg appearance of kidney was also found to be negative for ASF.

DISCUSSION

Jharkhand is a major pig farming state of India. It boasts a pig population of 12.8 million which is only second to Assam as per 2019 livestock census. As the state is in close proximity to north-eastern states, it is always susceptible to interstate crossover of disease. The etiological agent of ASF is African swine fever virus (ASFV), a member of family *Asfarviridae*, and Asfivirus varies in length between 170-193 kbp[7]. The occurrence of ASF in India was first reported from adjoining states of Arunachal Pradesh and Assam in 2020 as trans-boundary disease when virus crossed over from China[4].

The virus with its adaptability to replicate in its hosts

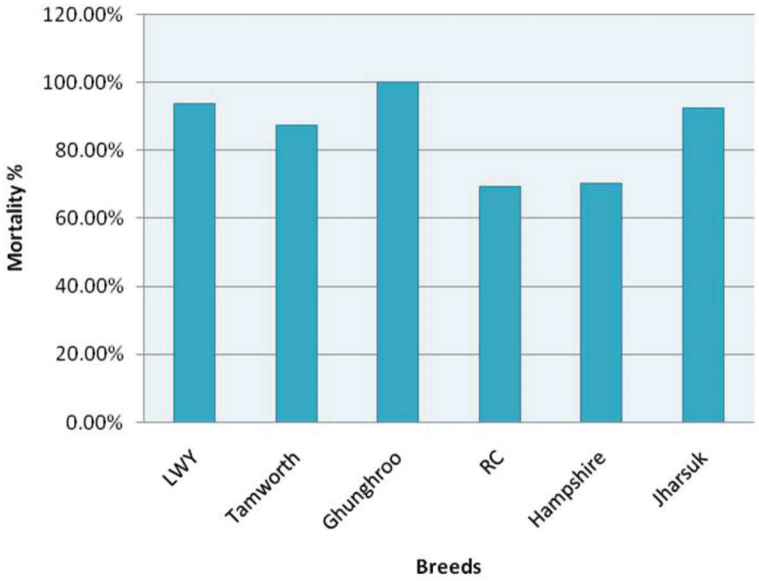

Fig. 14. Mortality percent of different breeds during the outbreak of ASF (Graph).

Table 3. Mortality percent of different breeds during the outbreak of ASF.

S.No.	Breed	Mortality percent
1.	LWY	93.69%
2.	Tamworth	87.50%
3.	Ghunghroo	100%
4.	RC	69.44%
5.	Hampshire	70.37%
6.	Jharsuk	92.59%

in the sylvatic cycle, poses special threat to the pig population of Jharkhand state due to its vast forest area and presence of wild boar susceptible to ASFV, thus increasing the possibility of recurrence of the disease in future as they also maintain the virus. Its capability to spread rapidly to different regions through wild life hosts, climatic conditions, pig management system and blatant disregard for biosecurity measures in rural population makes it a very dangerous pathogens for pig farming industry of Jharkhand and India. The entry of virus into the body cause viraemia in 3 days' followed by its presence in nasal and rectal fluid by 10-13 days' can subsequently infect the pen mates through inhalation or ingestion of infected material[7]. ASFV is usually transmitted between infected pigs by direct contact through oral-nasal route or through skin abrasions. Bleeding from natural orifices was a common clinical manifestation in the present outbreak, hence rapid spread within both the pig farms must have been through direct or indirect contact of pen mates within infected premises, through ingestion of infected material or indirectly through attendant. However, spread of virus from GPF, to IPF, located 500 meters apart with no direct connection must have been through aerosol route because virus can travel short distance through aerosol route[8,9]. The outbreak in Jharkhand was controlled by imposing strict ban on the movement of pigs in the state and in the locality subsequent to confirmation of the disease by ICAR-NISHAD, Bhopal, India. Massive awareness drive was taken up with establishment of protection and surveillance zone in an area of 3 km and 10 km, respectively.

Major gross lesion observed during the outbreak comprised of marked splenomegaly and lymphadenitis of hepatic and mesenteric lymphnodes with haemorrhagic and oedematous changes. Haemorrhages and congestive changes were also recorded in epicardium, endocardium, gastric mucosa, intestinal mucosa and lungs etc. The present findings are similar to the observation of[10]. Haemorrhagic and hyperaemic splenomegaly is considered as most typical lesion of ASF and the severity of lesion depends on virulence of the isolate[11,12].

Histopathologically, marked haemorrhagic and necrotic changes were observed in the spleen, lymphnodes, endocardium, epicardium, as well as in gastric and intestinal mucosa. Similar histopathological findings in ASF outbreak has been reported earlier[10,13]. Washed out appearance of lymphoid follicles was a major consistent change in lymph nodes and spleen, which makes the animal immunodeficient. Lymphoid cell depletion in the lymphoid organs during ASF has been recorded by Gomez-Villamandos[1]. Increased TNF-α, IL-1α, IL-1β and IL-6 cytokine expression has been reported in lymphoid organs along with increased serum level of TNF-α and IL-1β. These changes bring about apoptotic loss of lymphocytes as well as create cytokine storm with resultant disseminated intravascular coagulopathy (DIC) and consumption coagulopathy getting expressed as widespread petechial, ecchymotic and haemorrhagic lesions and accumulation of transudate in the body cavity[10]. TNFα is reported to play a key role in the pathology of ASF by inducing alteration in vascular permeability, coagulation and induction of apoptosis in uninfected lymphocytic population[14].

Higher depletion of cells in the lymphoid follicles is suggestive of higher virulence of the virus,[15,16] this confirms that highly virulent strain of ASFV prevailed in the state of Jharkhand. Mononuclear cell predominantly macrophage infiltration was commonly seen histopathologically in different organs. Lymphopaenia and lymphoid depletion in lymphoid organ during ASF has also been described[13]. The cause of cellular damage observed in lymphoid and other organs has been attributed to activation of macrophage triggered cytokine mediated interaction rather than virus induced[17].

The phagocytic activation of capillary endothelial cells with resultant hypertrophied endothelial cells culminates in obliteration of the capillary lumen, leading to increased intravascular pressure[18], loss of endothelial cells and exposure of the capillary basement membrane with resultant microthrombus formation, petechiations, ecchymosis and exudation of oedema fluid[18,19].

The liver in pig suffering from ASF mostly exhibited marked congestion and also multifocal periportal inflammatory cell infiltration, similar findings have been reported earlier[10,13,20-23]. These lesions are responsible for mottled appearance of liver. In the present study, prominent multifocal hepatic necrotic lesion was a consistent feature which has not been described by earlier workers. It might be due to highly virulent nature of the virus substantiated by the fact that haemorrhagic lesions too were quite extensive in heart, spleen, entero-hepatic lymph nodes, gastric mucosa and intestinal mucosa.

Reduced incidence of ASF in starter and grower pigs in the initial one month of outbreak could be attributed to less production of pro

Table 4. Chi-Square analysis of overall mortality in different breeds during the outbreak of ASF.

S.No.	Between groups	P value
1.	LWY x Tamworth	0.030^{NS} ($p<0.05$)
2.	LWY x Ghunghroo	0.017^{NS}
3.	LWY x Russian Charmukha	1.042^{NS}
4.	LWY x Hampshire	1.294^{NS}
5.	Tamworth x Ghunghroo	0.050^{NS}
6.	Tamworth x Russian Charmukha	0.265^{NS}
7.	Tamworth x Hampshire	0.266^{NS}
8.	Ghunghroo x Russian Charmukha	0.460^{NS}
9.	Ghunghroo x Hampshire	0.465^{NS}
10.	Russian Charmukha x Hampshire	0.0016^{NS}

**Significant at p< 0.01; *Significant at p<0.05; NS - Non-significant

inflammatory TNF cytokines including Leukotriene A, Leukotriene B and Fas Lig and probably due to less developed macrophage-monocyte system in the early life. However with age it reaches optimal functioning which might induce massive cytokine storm and heavy mortality in animals[24]. Contrary to our observations, higher resistance to ASFV in older pigs as compared to younger pigs has been described in pig breeds[25].

The mortality pattern in different breeds and varieties of pigs showed relative resistance to ASFV in Russian Charmukha, Hampshire, Tamworth and Jharsuk. Large White Yorkshire was found to be most susceptible. However, the variation in mortality rate between different breed and varieties of pig were non-significant. The gene associated with resistance to ASFV infection is termed as RELA (Reticuloendotheliosis viral oncogene homolog)[26]. The gene causes the immune system to over react as it detects the disease. Significant variation in the expression of RELA genes between resistant and susceptible pig is associated with host response to ASF infection. Another cause of resistance may be innate immune system and its ability to control viral replication resulting in reduced systemic infection. This might be the cause of variable susceptibility of different varieties of pigs. There is uncertainty regarding genome connected with virulence of ASFV and needs further exploration. The two ASFV virulence genes are UK and 23-NL located adjacent to each other in right variable region of the genome. Deletion of UK and 23-NL genes diminishes the virulence of ASFV without affecting virus replication in macrophages[27,28]. Previous findings suggest that genotype II isolates of the "Georgia 2007 type" strain prevalent in eastern and central Europe and recently in Asia are highly virulent and cause mortality in the range of 91-100%[29]. The nature of virus involved in Ranchi outbreak needs further exploration on above line of action.

In the Ranchi (Jharkhand) outbreak it has been observed that 100% tissues collected from dead animals were positive for ASF, whereas the positive percentage was only 33.3% when the samples from live animals during the outbreak were tested for ASF by molecular diagnostic technique. This makes the tissue collected from dead animals a better sample for diagnosis of ASF through molecular techniques in future studies. Another interesting finding was that the investigation done for diagnosis of ASF from surviving animals after one and half month of outbreak were all negative for ASF. This is contrary to the report of[30] which mentioned that survivors may develop persistent infection in susceptible tissues of the body and cause disease under favourable conditions such as immunosupression, transportation or irregular and under feeding to the animals.

Thus, it can be concluded that the lower age group showed significantly lower mortality than the other two age groups during the outbreak. More research is required to understand some of the pathogenetic mechanisms, including how ASFV modulates the host immune responses in different age groups which make them less susceptible to ASF. This appears to be the first report of ASF in Jharkhand.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the facilities provided by Dean, College of Veterinary Science and Animal Husbandry, Birsa Agricultural University, Director Institute of Animal Health Products, Ranchi and Pig Development Officer, Govt. Pig Farms, Ranchi Jharkhand to carry out the study.

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Indian J. Vet. Pathol., 48(1) : 26-34, 2024: DOI: 10.5958/0973-970X.2024.00005.1

RESEARCH ARTICLE

Ameliorative effect of *Withania Somnifera* and Shilajit on brain lesions and behaviour of hypothyroid rats

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Received: 12.8.2023; Accepted: 24.9.2023

ABSTRACT

The experiment was conducted to evaluate neurobehavioural alterations, histopathology and electron microscopic changes in brain of hypothyroidism induced rats. 21 days old weaned female *Sprague drawly* rats (96) were divided into 8 groups with 12 No. in each group. They were treated as Group 1: Euthyroid, Group 2: Hypothyroidism induced with methimazole @ 0.02%, Group 3: Hypothyroid + LT4, Group 4: *Withania somnifera* root extract control, Group 5: Hypothyroid + *Withania somnifera* root extract, Group 6: Shilajit control, Group 7: Hypothyroid + Shilajit and Group 8: Hypothyroid + *Withania somnifera* root extract + Shilajit, both @ 100 mg/Kg b. wt. At the end of 3 months, 6 rats from each group were sacrificed and brains were collected for histopathology and electron microscopic studies. The remaining rats were mated with euthyroid adult males and all the 48 pregnant rats were administered with treatments as above till the 17th day of gestation. The F_1 generation pups were reared till 21 days to study neurobehavioral parameters and were sacrificed to collect brain and thyroid for histopathology and electron microscopy studies. Histopathology and TEM sections of group II brains, in parent stock and F_1 generation revealed neuronal swelling, reduced nissil substance, satellitosis, neuronophagia and apoptosis. TEM revealed swollen nucleus, condensed mitochondria, loss of cristae and matrix, irregular discontinuous endoplasmic reticulum and loss of nuclear membrane. The present study showed neurological alterations in hypothyroidism model and usage of herbal drugs have beneficiary role in alleviating the effects of hypothyroidism.

Keywords: Electron microscopy, histopathology, hypothyroidism, methimazole, shilajit, *Withania somnifera*

INTRODUCTION

Thyroid status has emerged as a potential independent risk factor for the development of reversible cognitive impairment for the past two decades. Thyroid hormones have been demonstrated to play central roles in the development of central neural system, maintenance of normal neural functions, as well as the regulation of protein, fat and carbohydrate metabolism[1]. The nervous system and the endocrine system are involved intimately in maintaining homeostasis. Therefore, endocrine dysfunctions may lead to various neurologic manifestations. The neuroendocrine system which is made up of the nervous system and the endocrine system work together to maintain body functions[2]. Thyroid hormones have significant role in signaling of brain functions, somatic maturity in infants and metabolic activities in adults.

Deficiency of thyroid hormone causes a retardation of the development of the CNS and alteration in neurological development. Thus hypothyroid pups open their eyes late. Hypothyroidism in pregnant animals and humans causes alterations in neurological development[3]. Learning and memory are clearly impaired in animals following developmental hypothyroidism[4]. A deficiency of thyroid hormones during lactation, neonatal and juvenile periods could impair spatial learning and memory in the Morris water maze test[5]. Foetus from hypothyroid dam produces neurological deficits, as maternal thyroid hormone cannot reach the foetus like in normal condition thus leads to foetal hypothyroidism[6].

How to cite this article : Ramya, B., Kumar, A.A., Reddy, A.G., Lakshman, M. and Shivakumar, P. 2024. Ameliorative effect of *Withania Somnifera* and Shilajit on brain lesions and behaviour of hypothyroid rats. Indian J. Vet. Pathol., 48(1) : 26-34.

Herbal and herbomineral products are regularly used in Ayurveda; that are believed to be strengthen the body's defenses[7]. *Asphaltum punjabianum* (Shilajit) and *Withania somnifera* have been used for cognitive enhancement, memory-improving and neuro protective properties[8,9].

MATERIALS AND METHODS

If total of 21 days old weaned

Table 1. Serum thyroid profile in different groups of rats (Parent stock).

Groups (n=6)	T_3 (ng/dl) concentration	T_4 (µg/dl) concentration	TSH (µ IU/ml) concentration
Group 1	115.68±4.46^{b}	4.71±0.14^{b}	0.05±0.01^{a}
Group 2	101.15±4.06^{a}	3.01±0.24^{a}	5.91±0.45^{c}
Group 3	110.73±4.84^{b}	4.36±0.18^{b}	0.87±0.03^{b}
Group 4	118.03±4.69^{b}	4.79±0.17^{b}	0.03±0.007^{a}
Group 5	111.74±4.17^{b}	4.24±0.17^{b}	1.04±0.03^{b}
Group 6	116.62±5.02^{b}	4.69±0.19^{b}	0.04±0.009^{a}
Group 7	106.78±4.71^{a}	3.45±0.12^{a}	1.91±0.16^{b}
Group 8	113.19±4.76^{b}	4.31±0.21^{b}	0.51±0.12^{b}

Means with different superscripts differ significantly ($p<0.05$), One way ANOVA (SPSS:15)

female *Sprague-drawly* rats (96) were procured from Mahaveera enterprises, Hyderabad. Rats were maintained in modern animal house attached to the Department of Veterinary Pharmacology & Toxicology with 12 h - 12 h dark and light cycle at a temperature of 22-24^{O}C. Rats were housed in solid bottom polypropylene cages, placed on commercial standard pellet feed and provided water *ad libitum*. Rats were kept for acclimatization for five days prior to start of the experiment. The study was carried out with prior approval of Institutional Animal Ethics Committee (IAEC), College of Veterinary Science, Rajendranagar (I/6/16/05-01-16).

All the rats were divided into 8 groups with 12 in each group and the experiment was conducted for a period of 3 months as per the schedule given below:

Group 1: Euthyroid

Group 2: Hypothyroidism induced with Methimazole @ 0.02%

Group 3: Hypothyroid + LT_4

Group 4: *Withania somnifera* root extract control @ 100 mg/Kg b. wt.

Group 5: Hypothyroid + *Withania somnifera* root extract @ 100 mg/Kg b. wt.

Group 6: *Shilajit* control @ 100 mg/Kg b. wt.

Group 7: Hypothyroid + *Shilajit* @ 100 mg/Kg b. wt.

Group 8: Hypothyroid + *Withania somnifera* root extract @ 100 mg/Kg b. wt. + *Shilajit* @ 100 mg/Kg b. wt

Thyroid profile was estimated from sera of blood collected at the end of third month by Radio Immuno assay employing DiaSorin S.p.A. kits, USA. At the end of 3 months, 6 rats from each group were sacrificed and brains and thyroids were collected in suitable preservatives for histopathological and electron microscopic studies. The tissues of brain were stored at -20^{0}C for further estimation of GSH[10], TBARS[11] and protein carbonyls[12] in homogenates.

Remaining 48 female rats (6 from each group) were mated with euthyroid adult (above 3 months) males (procured from Mahaveera Enterprises, Hyderabad) and above treatment protocol continued up to 17th day of gestation.

Pups were reared till the weaning age (21 days) to study the neurobehavioral parameters such as elevated plus maze[13] and Morris water maze[14]. On sacrifice of the pups, representative pieces of brain and thyroid gland were collected in suitable preservatives for electron microscopy[16] (Hitachi, H-7500) and for histopathology. Paraffin embeded sections were stained with Hematoxylin and Eosin (H & E) stain[15].

Table 2. Oxidative stress parameters of different groups of rats (Parent stock).

Groups (n=6)	TBARS concentration (n moles of MDA released/mg protein)	GSH concentration (n moles/mg protein)	Protein carbonyls concentration (n moles/mg protein)
Group 1	24.76±2.61^{a}	40.26±3.79^{bc}	1.92±0.12^{a}
Group 2	38.17±2.80^{c}	27.11±2.94^{a}	7.52±0.34^{c}
Group 3	26.22±3.10^{a}	38.23±4.07^{bc}	2.71±0.04^{a}
Group 4	23.78±2.11^{a}	47.15±4.75^{c}	1.09±0.13^{a}
Group 5	28.16±1.91^{ab}	36.24±3.14^{b}	3.01±0.23^{a}
Group 6	24.08±1.86^{a}	43.15±4.75^{bc}	1.71±0.21^{a}
Group 7	31.27±2.63^{b}	34.67±2.94^{b}	4.94±0.27^{b}
Group 8	27.09±1.78^{ab}	38.15±3.19^{bc}	2.04±0.11^{a}

Means with different superscripts differ significantly ($p<0.05$), One way ANOVA (SPSS)

The data obtained from the study were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) version 15.0. Differences between means were tested using Duncan's multiple comparison tests and significance level was set at 0.05.

RESULTS

Thyroid profile

In thyroid Profile, the serum T_3 and T_4 hormone concentration (ng/dl) of the group 2 was significantly ($p<0.05$) lower than group 1 while the values in groups 3, 4, 5, 6 and 8 were comparable to group 1. T_3 concentration in group 7 was significantly ($p<0.05$) lower than group 1. The serum TSH hormone concentration (µ IU/ml) of the group 2 was significantly ($p<0.05$) higher than group 1 and was significantly lower in groups 3 to 8 than group 2 (Table 1).

Oxidative stress

Oxidative stress parameters like the concentration of Thiobarbituric acid reacting substances (n moles MDA/mg protein) showed a significant ($P < 0.05$) rise in group 2 as compared to groups 1, 3, 4 & 6. The groups 5 and 7 showed a significant ($p<0.05$) decrease in TBARS concentration as compared to group 2.

The concentration of GSH (n moles/mg protein) decreased significantly ($p<0.05$) in group 2 as compared to group 1. The groups 3 to 8 showed a significant ($p<0.05$) increase in GSH concentration as compared to group 2.

The concentration of protein carbonyls (n moles/mg protein) increased significantly ($P < 0.05$) in group 2 as compared to groups 1 while groups 3 to 8 revealed a significant ($P < 0.05$) decrease as compared to group 2 (Table 2).

Neurobehavioral studies

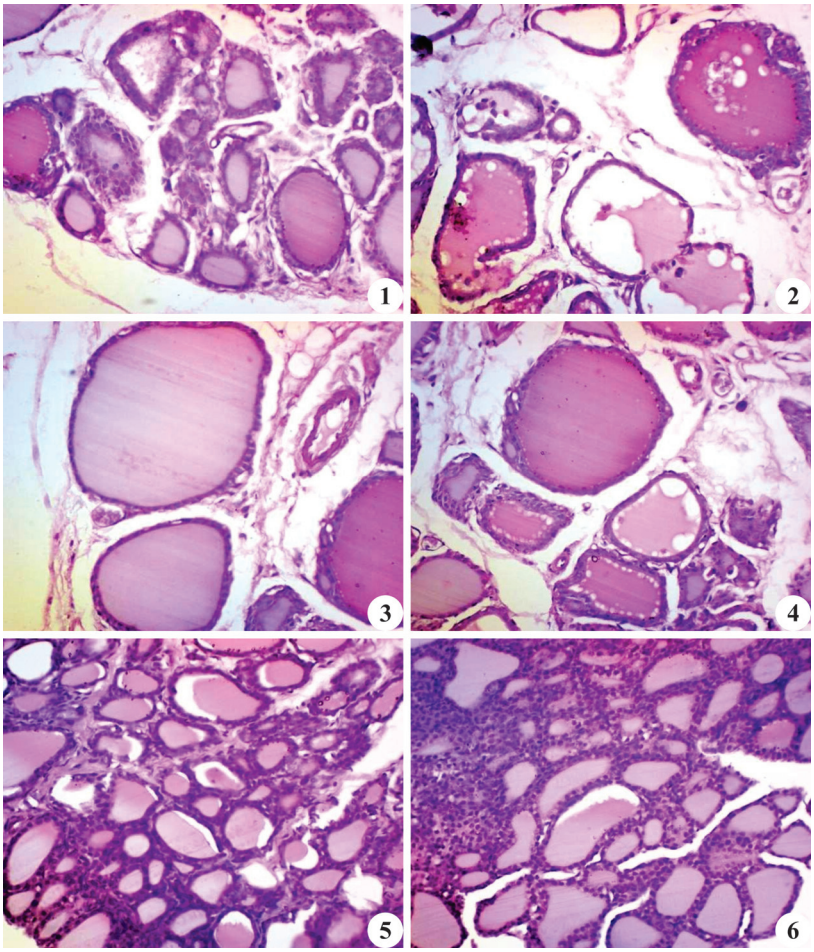

Fig. 1. Group II - Section of thyroid gland showing altered shape and size of follicles with presence of C cells and few parafollicular cells (H&E, X400); **Fig. 2.** Group II - Section of thyroid gland showing vacuolation, some follicles contained varied amounts of colloid, the others were devoid of colloid (H&E, X400); **Fig. 3.** Group V - Section of thyroid gland showing normal follicles (H&E, X400); **Fig. 4.** Group VII - Section of thyroid showing mild changes in follicles (H&E, X400); **Fig. 5.** Group II - Section of thyroid gland showing altered follicles with variation in shape and size, few follicles contained less colloid, few were devoid of colloid and few others were with varied amounts of colloid (H&E, X400); **Fig. 6.** Group V - Section of thyroid showing changes were mild with almost comparable with that of normal control groups (H&E, X400).

Table 3. Performance on Elevated Plus Maze in different groups of rats (F_1 generation).

Groups (n=6)	No. of entries		Duration of time spent (sec)		
	Open arm	Closed arm	Centre of the maze	Open arm	Closed arm
Group 1	6.71±0.64^{a}	11.76±0.54^{c}	49.66±5.12^{a}	56.22±4.14^{a}	194.12±7.87^{c}
Group 2	11.76±1.64^{b}	3.09±0.24^{a}	65.22±4.69^{c}	189.23±7.09^{c}	45.55±3.29^{a}
Group 3	8.06±1.27^{ab}	9.88±1.07^{bc}	54.29±4.44^{b}	102.71±6.22^{b}	143.00±4.24^{b}
Group 4	5.02±0.54^{a}	13.36±1.26^{c}	47.83±3.49^{a}	58.66±3.79^{a}	193.51±5.86^{c}
Group 5	9.71±1.03^{b}	7.72±0.64^{b}	45.87±3.16^{a}	96.03±5.64^{b}	158.10±5.07^{b}
Group 6	6.34±0.54^{a}	11.51±1.26^{c}	51.66±3.17^{b}	63.02±3.24^{a}	185.35±4.79^{c}
Group 7	10.09±1.37^{b}	9.16±1.78^{bc}	56.21±3.86^{b}	106.27±5.07^{b}	137.52±4.51^{b}
Group 8	7.81±1.33^{ab}	10.29±1.55^{bc}	59.15±4.31^{bc}	97.24±5.31^{b}	143.61±4.07^{b}

Means with different superscripts differ significantly ($p<0.05$), One way ANOVA (SPSS:15)

In elevated plus maze, the number of entries in open arm revealed a significant ($p<0.05$) increase in group 2 as compared to groups 1, 4 and 5 while group 3 and 8 showed a decrease compared to group 2. The treatment groups 5 and 7 revealed a significant ($p<0.05$) increase as compared to group 1. The number of entries in to the closed arm, duration of time spent (sec) in centre of maze, open arm and closed arm revealed a significant ($p<0.05$) decrease in group 2 as compared to groups 1 and 3 to 8. In Morris water maze, the time taken to identify the platform (sec) in probe trial and the total distance travelled (cm) in revealed that group 2 took significantly ($p<0.05$) more time than groups 1 and 3 to 8.

Gross pathology

No gross lesions of pathological significance were observed in any of the organs except very mild reduction in size of thyroid gland in group II rats but was not appreciable grossly in parent stock might be due to immunosuppressive action of induced drug.

Histopathology

Thyroid gland (Parent stock)

In groups 1, 3, 4 and 6 no lesions of pathological significance were observed. The section of thyroid in group II, showed variation in shape and size of follicles with varied amounts of colloid and few with devoid of colloid (Figs. 1-2). In groups 5 and 7 the changes were mild with almost comparable with that of normal control groups (Figs. 3-4) and in group 8, the changes could not be seen and almost comparable with that of normal control groups.

Thyroid gland (F_1 Generation)

In groups 1, 3, 4 and 6 no lesions of pathological significance were observed. The section of thyroid in group 2 showed altered follicles with variation in shape and size, few follicles contained less colloid, few were devoid of colloid and few others were with varied amounts of colloid (Fig. 5). In groups 5 and 7, the changes were mild with almost comparable with that of normal control groups (Fig. 6) and in group 8 the changes were almost comparable with that of normal control groups.

Brain (Parent stock)

Section of brain in groups 1, 3, 4 and 6 did not show any lesions of pathological significance. In group II, neuronal swelling, elongation, vacuolation, degeneration, gliosis and congestion with reduced nissil substance (Figs. 7-9). In groups 5 and 7, the changes observed were moderate to mild in nature (Figs. 10-11) and in group 8, changes no lesser of pathological significance and were comparable to that of normal control groups.

Brain (F_1 Generation)

Table 4. Performance on Morris Water Maze in different groups of rats (F_1 generation).

Groups (n=6)	Escape Latency (in seconds)			Probe trial (sec)	Total distance travelled (cm)
	EL1	EL2	EL3		
Group 1	46.22±3.79	41.37±3.64	37.22±3.14	37.61±3.46^{a}	501.41±12.66^{a}
Group 2	53.76±4.61	51.48±4.06	49.34±3.36	52.04±3.48^{c}	767.44±14.07^{c}
Group 3	51.93±3.88	49.22±3.15	43.24±3.12	42.75±3.61^{a}	631.04±10.68^{b}
Group 4	44.87±4.12	41.71±4.21	40.31±3.61	40.36±3.81^{a}	505.22±11.14^{a}
Group 5	50.34±2.97	49.34±3.06	47.34±3.42	46.11±3.49^{ab}	662.48±12.33^{b}
Group 6	48.91±3.22	49.47±3.37	41.61±3.14	40.77±3.61^{a}	572.41±11.23^{ab}
Group 7	48.93±3.74	51.31±4.05	48.58±4.27	48.47±4.31^{b}	684.23±12.41^{bc}
Group 8	52.99±3.82	50.97±4.71	49.24±3.68	46.63±4.78^{ab}	651.84±11.73^{b}

Means with different superscripts differ significantly ($p<0.05$), One way ANOVA (SPSS:15)

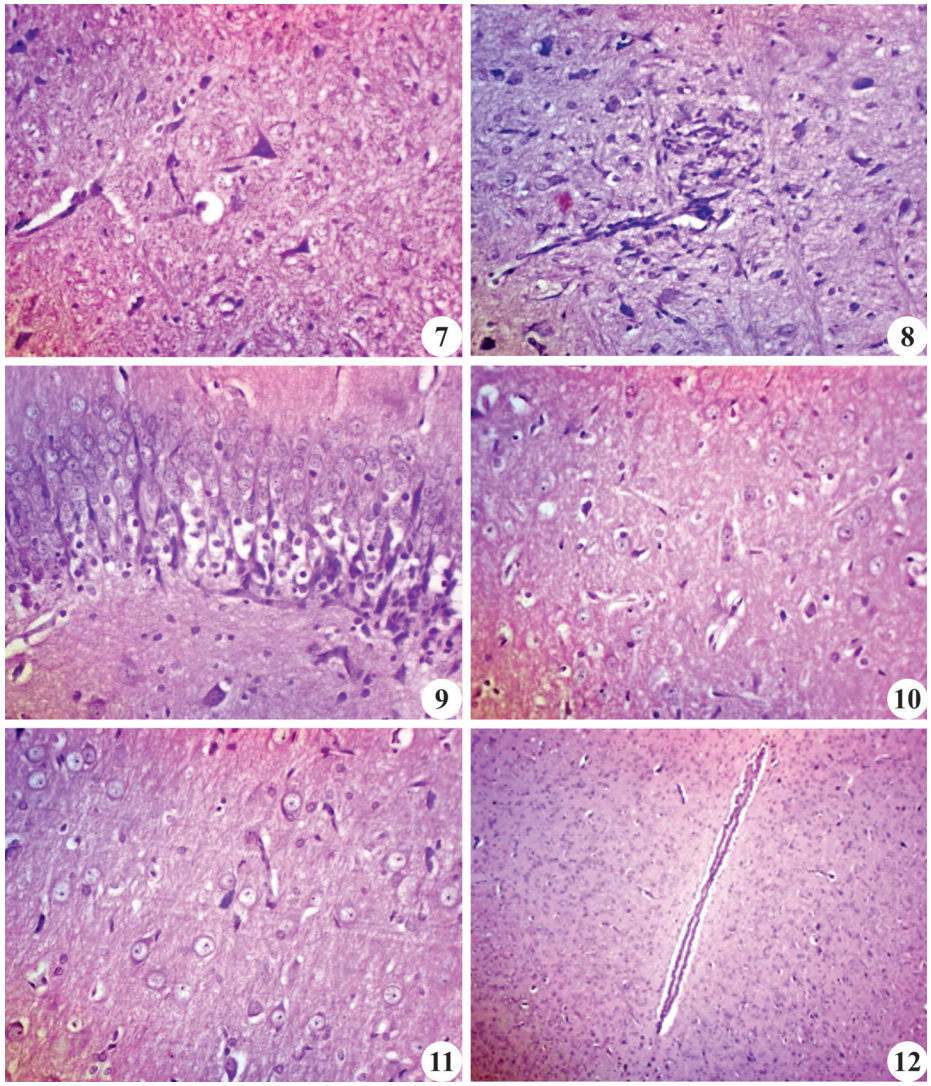

Fig. 7. Group II - Section of brain showing neuronal swelling, elongation, vacuolation and degeneration (H&E, X400); **Fig. 8.** Group II - Section of brain showing gliosis and congestion (H&E, X400); **Fig. 9.** Group II - Section of brain showing reduced nissil substance (H&E, X400); **Fig. 10.** Group V - Section of brain showing moderate changes (H&E, X400); **Fig. 11.** Group VII - Section of brain showing mild changes (H&E, X400); **Fig. 12.** Group II - Section of brain showing congestion (H&E, X100).

Section of brain in groups 1, 3, 4 and 6 did not show any lesions of pathological significance. In group II, congestion, neuronal swelling, reduced nissil substance, satellitosis, neurofibrillary degeneration and gliosis was noted (Figs. 12-13). In groups 5 and 7 mild neuronal degeneration and apoptosis were noted (Figs. 14). In group 8, changes no lesser of pathological significance and were comparable to that of normal control groups.

Electron microsocpy

Thyroid gland (parent stock)

The sections of thyroid gland from rats of all groups were subjected to transmission electron microscopy. The lesions in group 2, showing mitochondria lost their morphology and dilated endoplasmic reticulum, thyrocytes were under process of necrosis, increase in inter-follicular connective tissue septum, mitochondria lost their morphology and dilated endoplasmic reticulum was noted (Figs. 15-16). In groups 5 and 7, sections showed loss of junction, variation in shape and size of nucleus, margination of chromatin and disintegrated nuclues with vesicular cytoplasm (Fig. 17).

Thyroid gland (F_1 Generation)

The section of groups 1, 3, 4 and 6 showed normal interfollicular junction with regular follicular epithelium and nucleus. In group 2, swollen nucleus, increase in perinuclear space, moderate to severe margination of chromatin, dilation of nuclear pore and inter follicular junction was noted. Further vesicular cytoplasm, variation in size and shape of nucleus with dilated endoplasmic reticulum and interfollicular junction was observed (Figs. 18-19). In group 8, mild swollen nucleus with nucleolus, mild margination/diffused chromatin

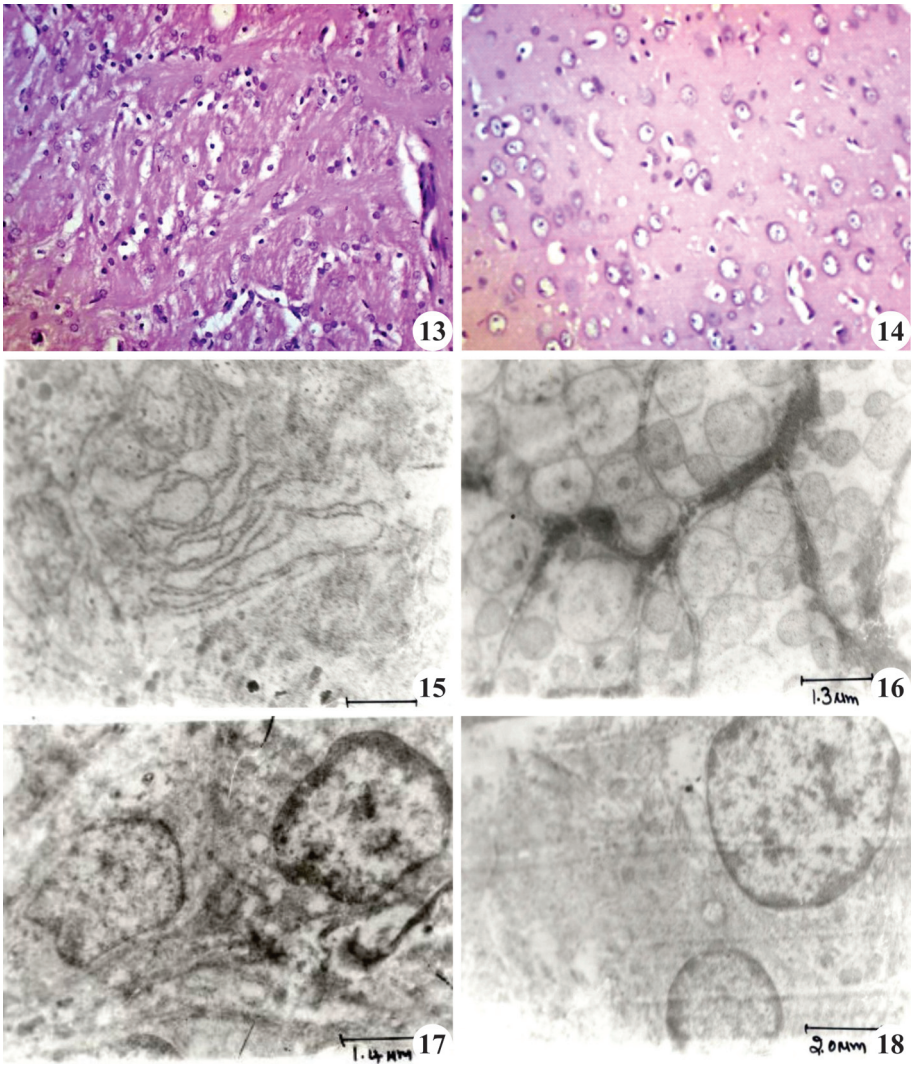

Fig. 13. Group II - Section of brain showing neurofibrillary degeneration and gliosis in group II (H&E, X400); **Fig. 14.** Group V - Section of brain showing mild neuronal degeneration (H&E, X400); **Fig. 15.** Group II - TEM section of thyroid gland showing mitochondria lost their morphology and dilated endoplasmic reticulum. Uranyl acetate lead citrate, X23160; **Fig. 16.** Group II - TEM section of thyroid showing thyrocytes under process of necrosis, increase in inter-follicular connective tissue septum. Uranyl acetate lead citrate, X15440; **Fig. 17.** Group VII - TEM section of thyroid showing loss of junction, variation in shape and size of nucleus, margination of chromatin and disintegrated nuclues with vesicular cytoplasm. Uranyl acetate lead citrate, X13510; **Fig. 18.** Group II - TEM section of thyroid gland showing swollen nucleus, increase in perinuclear space, moderate to severe margination of chromatin, dilation of nuclear pore and inter follicular junction. Uranyl acetate lead citrate, X9650.

with numerous electron dense mitochondria and almost normal features were observed (Fig. 20).

Brain (F_1 Generation)

The section of groups 1, 3, 4 and 6 showed no lesions of pathological significance. In group II, swollen nucleus, margination of chromatin, condensed mitochondria, loss of cristae and matrix, vesicular cytoplasm, irregular discontinuous endoplasmic reticulum and loss of nuclear membrane and irregular shrunken neuron and swollen neuron with thin myelin sheath were noticed (Figs. 21-22). In groups 5 and 7, granular cell layer with numerous neurons varying in shape and size with thick myelin sheath were observed (Fig. 23). In group 8, mild to normal features of reconstruction like swollen nucleus with margination, perinuclear space and vesicles with indistinct cytoplasm and organelles was noted (Fig. 24).

DISCUSSION

The significant ($p<0.05$) decrease in serum T_3 and T_4 hormone concentration (ng/dl) and significant ($p<0.05$) increase in TSH hormone concentration (µ IU/ml) of the group 2 might be due to methimazole's inhibition of the enzyme thyroperoxidase, that acted in thyroid hormone synthesis by oxidizing the anion iodide (I^-) to iodine (I_2), hypoiodous acid (HOI), enzyme linked hypoiodate (EOI) facilitating iodine's addition to tyrosine residues on the

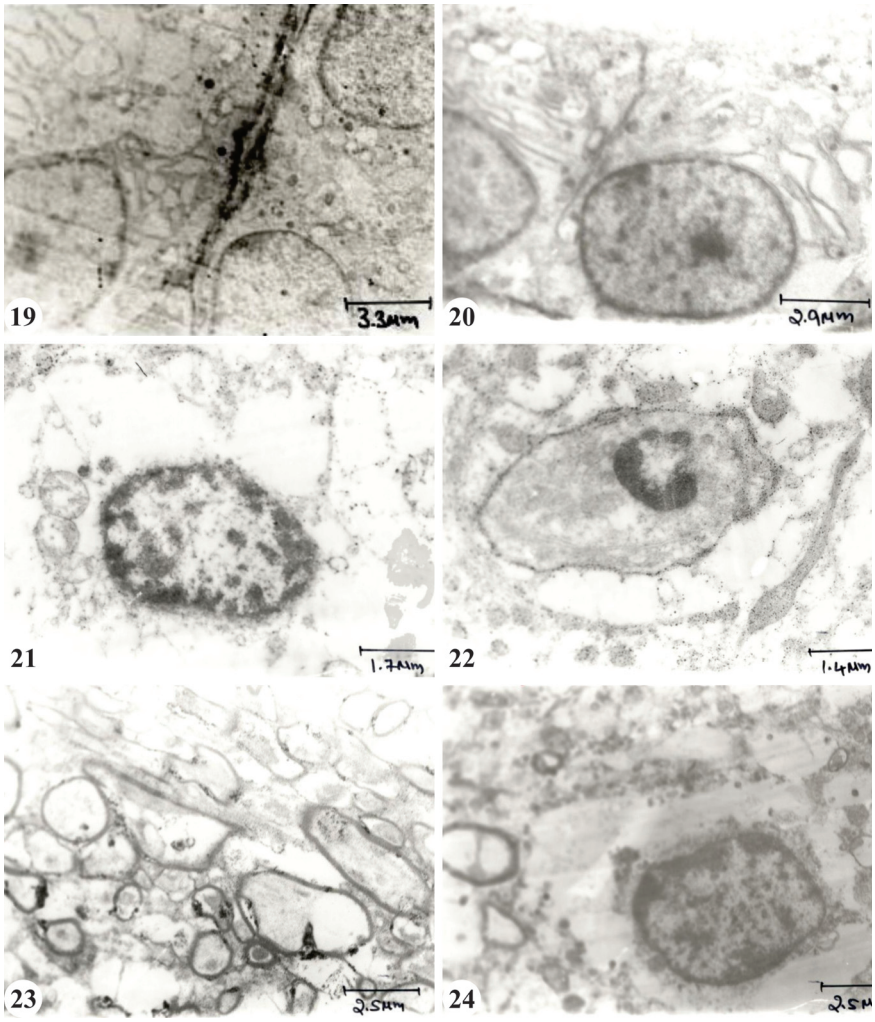

Fig. 19. Group II - TEM section of thyroid gland vesicular cytoplasm, variation in size and shape of nucleus with dilated endoplasmic reticulum and interfollicular junction. Uranyl acetate lead citrate, X28950; **Fig. 20.** Group VIII - TEM section of thyroid gland showing mild swollen nucleus with nucleolus, mild margination/diffused chromatin with numerous electron dense mitochondria and almost normal features. Uranyl acetate lead citrate, X6755; **Fig. 21.** Group II - TEM section of brain showing swollen nucleus, margination of chromatin, condensed mitochondria, loss of cristae and matrix, vesicular cytoplasm, irregular discontinuous endoplasmic reticulum and loss of nuclear membrane. Uranyl acetate lead citrate, X11580; **Fig. 22.** Group II - TEM section of brain showing irregular shrunken neuron and swollen neuron with thin myelin sheath. Uranyl acetate lead citrate, X13510; **Fig. 23.** Group V - TEM section of brain showing granular cell layer with numerous neurons varying in shape and size with thick myelin sheath. Uranyl acetate lead citrate, X7720; **Fig. 24.** Group VIII - TEM section of brain showing mild to normal features of reconstruction like swollen nucleus with margination, perinuclear space and vesicles with indistinct cytoplasm and organelles. Uranyl acetate lead citrate, X7720.

hormone precursor thyroglobulin, a necessary step in the synthesis of triiodothyronine (T_3) and thyroxine (T_4). The decrease in T_3 and T_4 through negative feedback mechanism might have stimulated pituitary to release more thyroid stimulating hormone[17].

Increase in thyroid hormone synthesis in group 5 rats might be due to adaptogenic nature of Ashwagandha, that worked well with the hormones of the endocrine system and brings balance to the thyroid hormonal level. Immunomodulation effect of herb has a stimulatory effect on a sluggish thyroid and increased serum T_4 concentration[18]. *Shilajit* improved thyroid profile by controlling thyroid gland function through it's adaptogenic activity[19].

Oxidative stress parameters like TBARS and protein carbonyls were significantly ($p<0.05$) increased and GSH was decreased significantly ($p<0.05$) in group 2 rats. This might be due to hyperlipidemia in hypothyroidism that increased lipid peroxidation and hydrolyses of lipid peroxides which resulted in decrease of paraoxonase (PON1), antioxidant enzyme that peroxides in oxidized lipoproteins and caused increase in oxidative stress because of inverse association of enzyme with oxidative stress. Further, increased aldose reductase activity reduces NADPH and as a consequence there will be increase in reactive oxygen species that are responsible

for increase in TBARS and protein carbonyls[20]. Decreased GSH levels in the present study might be due to reduced activity of glucose 6-phosphate dehydrogenase (G6PD) which catalyses the nicotinamide adenine dinucleotide phosphate (NADPH) and acts as the principal intracellular reductant in the cells that is necessary for normal coupling of GSH[21]. The findings are also in correlation with the histopathological and electronmicroscopy of the present study.

In *Withania somnifera* treated group, a significant decrease and increase in levels of TBARS, protein carbonyls and reduced GSH respectively in lipid peroxidation might be due to increase in the efficacy of the antioxidant system of the cells by stimulating one or the other enzymes or increased activity of antioxidative enzymes that might be the secondary effects of plant extract induced increase in T_4 concentrations[22]. Antioxidant activity of *Shilajit* might be due to presence of resonance stabilized soft-spin semiquinone free radicals in it, that produce free radical scavenging and antioxidant effects against paramagnetic nitric oxide, SO_3-and OH radicals which might have influenced the present results of the study[8].

The efficiency of neurobehavioral studies with elevated plus maze in 21 day old pups born to methimazole treated rats in the present study was significantly ($p<0.05$) lower than other groups might be due to altered hippocampal formation and connectivity of the basic hippocampal trisynaptic loop that affect on density, percentage, ratio and size of vesicular glutamate transporter type 1 glutamatergic immunoreactive (VGluT1-ir) and vesicular inhibitory amino acid transporter GABAergic immunoreactive (VGAT-ir) boutons of the somatosensory cortex and hippocampal formation in developmental and early postnatal hypothyroidism in rats. Thus the increase in percentage of time spent in open arms in the present study might be due to deteriorated perception in gestation. Further, hypothyroid rats might have lost orientation and the memory. This can be explained as the risk-taking behavior of pups, so that they walk until the end of the open arm and fall. Impaired spatial learning and memory loss might also have altered their behavior in the elevated open arms[23].

Ashwagandha might have acted as anxiolytic by reducing the levels of tribulin, an endocoid marker of clinical anxiety and corticotrophin in the brain thus improving neurobehavioual efficiency[24]. Group 7 rats showed reduction in open arm entries, in relation to total arm entries, provided a measure of fear induced inhibition of exploratory activity that attenuated by anxiolytic activity of *shilajit*. Further, *shilajit* might have inhibited 5-hydroxytryptamine and 5-hydroxyindole acetic acid concentrations thereby the levels of dopamine, homovanillic acid and 3, 4-dihydroxyphenyl-acetic acid concentrations were increased, with insignificant effects on noradrenaline and 3-methoxy-4- hydroxyphenylethylene glycol levels that lead to increase in dopaminergic activity and might be responsible for neurochemical effects[25].

The decreased efficiency in Morris water maze might be due to alterations in hippocampal development and function that might have effected memory and learning as contributions of thyroid hormone deficiency[26]. The rats of group 2 travelled more distance to identify the platform in the probe trial[5, 27]. Chronic and perinatal hypothyroidism both might affect neuromotor competence, locomotor activity, cognitive performance and might have delayed neuromotor development, especially before weaning. After weaning, the negative effects on muscle strength, motor coordination and balance were greatly reduced, thus the delay in physical development and lag in cerebellar development may affect neuromotor proficiency and neuromotor impairment respectively[28]. Ashwagandha treatment enhanced efficiency in Morris water maze might be due to improvement of memory retrieval by the involvement of cholinergic mechanisms or by the GABA receptor inhibition and action on Ach receptors enhancing learning and memory improved short-term memory and anxiolytic action[29]. *Shilajit* is an immunostimulant and has been found to be very effective in treating immune and nervous disorders by inhibiting the degradation of neurons which was either by hypoxia or by free radicals or toxins in the brain, but has less affect than *Withania somnifera*[19].

In groups 5 and 7 improvement in the lesions of brain was observed and in group 8, no lesions were recorded. These findings might be due to anti stress and reduced antioxidant properties of *Withania somnifera* and *shilajit* and in combination[30].

Increase in thyroid hormone synthesis in group 5 rats might be due to adaptogenic nature of ashwagandha, that worked well with the hormones of the endocrine system and brings balance to the thyroid hormonal level. Immunomodulation effect of herb has a stimulatory effect on a sluggish thyroid and increased serum T_4 concentrations[18]. *Shilajit* improved thyroid profile by controlling thyroid gland function through it's adaptogenic activity[19]. *Withania somnifera* and *shilajit* improved thyroid gland function majorly by decreasing the thyroid stimulating hormone, responsible for ultrastructural changes in thyroid gland. These changes might be due to endocrine stimulating action of the herbs[8,31].

CONCLUSION

1. Neurobehavioral, histopathological and ultrastructural alterations in the present study indicated brain damage

at the molecular level that lead to free radicals release.

2. Administration of *Withania somnifera* and *Shilajit* individually and in combination, alleviated the deleterious effects caused by hypothyroidism due to their antioxidant and endocrine stimulant properties.
3. Synergistic action of herbs in ameliorating the effects of hypothyroidism is far better than administration of individual herbs.

Keeping this in view, further advanced studies can be advocated at molecular as well as by using different doses of herbs to obtain best results in combating the side effects of hypothyroidism which would be beneficial for animals as well as in humans.

ACKNOWLEDGEMENTS

The authors are grateful to DST for providing financial support for research and thankful to the University for providing facilities.

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Indian J. Vet. Pathol., 48(1) : 35-40, 2024: DOI: 10.5958/0973-970X.2024.00006.3

RESEARCH ARTICLE

Correlation of pendrin expression with ^{131}Iodine uptake in post-NaI treated thyroid cancer animal model

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Received: 5.9.2023; Accepted: 26.9.2023

ABSTRACT

Radioactive iodine (RAI) refractoriness develops in the background of a loss of thyroid differentiation features representing major therapeutic challenges in thyroid cancer management. Several studies have reported decreased or even loss of pendrin (SLC26A4 gene) expression in thyroid cancers, signifying the role of pendrin in the impaired ability of thyroid cancer cells for uptake and concentration of iodine. As acute iodide treatment has been shown to increase *SLC26A4* mRNA, the study of pendrin expression in malignant tissues is of interest. Hence, we focused on evaluating the pendrin expression and ^{131}I uptake in an N-bis-(2-hydroxypropyl) nitrosamine (DHPN) induced thyroid cancer model in Wistar rats followed by a single dose of excess sodium iodide (NaI) treatment to see if any change occurs in the RAI uptake. ^{131}I uptake by scintigraphy showed thyroid standard uptake values of 10.16±1.6 and 13.13±0.115 in DHPN-treated and control groups respectively at 24 h. However, post-NaI treatment decreasing patterns at 24 h, 48 h and 72 h were observed in a few animals similar to the control. However, the rising pattern of ^{131}I uptake was also observed in a few DHPN-induced thyroid cancer animals compared to the control. In conclusion, further extensive research is warranted to corroborate the role of NaI in the expression of pendrin in conjunction with animal species and age, along with the dose and exposure time of NaI. In addition, the expression and quantification of pendrin at the molecular level could be potentially used to enhance its utility in the management of RAI refractory human thyroid cancer patients.

Keywords: Follicular cells, immunohistochemistry, ^{131}Iodine uptake, N-bis-(2-hydroxypropyl) nitrosamine, pathology, pendrin, radioactive iodine, sodium iodide, thyroid cancer, Wistar rat

How to cite this article : Gholve, C.S., Shete, Y.H., Rakshit, S., Basu, S., Kulkarni, S.P. and Baghel, N.S. 2024. Correlation of pendrin expression with ^{131}Iodine uptake in post-NaI treated thyroid cancer animal model. Indian J. Vet. Pathol., 48(1) : 35-40.

INTRODUCTION

Sodium iodide symporter (NIS) and pendrin, functioning as iodide (I-) transporters, are proteins found principally, but not exclusively, in the thyroid tissue. Pendrin, a 110 kDa glycoprotein, encoded by the Pendred syndrome gene (PDS), is a member of the *SLC26A4* gene family. It is an anion transporter that is predominantly expressed in the inner ear, thyroid and kidney. In thyroid cells, pendrin is involved in apical iodide efflux[1]. The occurrence and level of pendrin expression and iodide efflux are regulated by TTF 1, TSH and thyroglobulin, while iodide itself does not have a major effect on pendrin gene expression[2]. On the contrary, Calil-Silveira *et al*.[3] showed that acute iodide treatment increased *SLC26A4* mRNA content, in both *in vitro* and *in vivo* models. Although pendrin is suggested to be an apical iodide transporter in the thyroid, the expression and localization of pendrin in diseased thyroids have not been adequately investigated[4]. The diagnostic and therapeutic uses of radioiodine in the management of thyroid cancers, however, are often hindered by the decreased ability of the thyroid cancer cells to uptake and concentrate iodine. This represents a major therapeutic challenge in thyroid cancer management. Hence, presently treatment tactics for radioactive refractory (RAI-R) thyroid cancer focus on novel approaches to re-differentiate thyroid cancer cells to restore the responsiveness to radioiodine administration by increasing the ^{131}I uptake. In this direction, various clinical trials are being conducted using different redifferentiating compounds in the preclinical experimental models of thyroid cancer as well as RAI-R DTC[5].

Several studies have reported decreased or even absent expression of *SLC26A4* in many thyroid tumors[6-10] demonstrating a pathological role of pendrin in the impairment of the iodide-concentrating mechanism of thyroid cancer cells. Therefore, the study of pendrin expression in pathological thyroid tissues, particularly in cancerous tissues, is of interest due to its iodide translocating activity, which serves as one of the iodide suppliers for organification processes. Our earlier animal studies have revealed the important role of pendrin in the uptake of ^{131}I in the thyroid follicles[11]. As acute iodide treatment has been shown to increase *SLC26A4* mRNA[3], the study of pendrin expression in malignant thyroid tissues

is of interest. The present work is a preclinical study that attempts to evaluate the expression of pendrin in a chemically induced thyroid cancer animal model in Wistar rats and to observe if any change in ^{131}I uptake, post-NaI treatment used for redifferentiation.

MATERIALS AND METHODS

Treatments

All the animal experiments were carried out as per the protocols/guidelines previously approved by the Committee for Control and Supervision of Experimental Animals (CPCSEA), India. The project was approved by Bhabha Atomic Research Centre, Animal Ethics Committee, Mumbai (Approval No. BAEC/27/18). A total of 18 male (270-280 g) 8-week-old healthy inbred Wistar strain rats were kept on ad libitum of commercial pelleted feed and filtered drinking water and maintained at a controlled temperature of 23±1^{O}C, humidity of 55±5%, in a 12h light/12h dark cycle. The animals were grouped into 2 groups based on their body weights. Group 1 (treatment group animals, n=12) and Group 2 (control animals, n=6) animals. Group 1 (treatment group) was injected with DHPN obtained from MedChem, UK (catalog number 53609-64-6) at a dose rate of 83 mg/animal by i/p route, fortnightly, for 4 months, whereas Group 2 (control group) animals received only saline. Post-treatment animals were subjected to tumor development analysis.

Thyroid scintigraphy

After a period of 16 weeks, Group 1 (DHPN treatment, n=12) and Group 2 (control, n=6) animals were subjected to the analysis of metabolic alteration in the thyroid gland by giving 296 KBq ^{131}I (8 µCi) orally and thyroid uptake and imaging was performed at 24h followed by excess NaI (2 mg NaI in 0.5 ml NaCl 0.9%) treatment with 2h, 24h, 48h and 72h uptake. The analysis of the uptake was done by a camera-based method with reference to the ^{131}Iodine standards source. Animal imaging was performed under Ketamine (80 mg/kg body weight) and Xylazine (5 mg/kg body weight) by i/p route for further studies. Ketamine and Xylazine are used as mild sedatives and muscle relaxants during imaging which was calculated and administered to the animals during the imaging considering further survival important in these animals to carry out the studies. Post-imaging the animals recover to normal state very fast. The uptake analysis was done with the help of software for the evaluation of disease initiation and its propagation[12]. All the radioactivity-related experiments were carried out under the supervision of the Radiation Safety Officer.

Histopathology of thyroid sections

At the end of 20 weeks, the terminal sacrifice of the animals was done by exsanguination, under ether anesthesia and thyroid tissues along with the other vital organs were collected for histopathology and immunohistochemistry. The bilateral thyroid lobes were excised and fixed in 10% neutral buffer formaldehyde and routinely embedded in paraffin. Further, tissue sections of 3-5µm thickness were prepared and stained with hematoxylin and eosin (H&E) stain. The histopathological changes in the thyroid gland were observed by light microscopic analysis. As per the published standard guidelines, focal proliferative lesions of follicular epithelial cells were classified in H&E-stained sections as focal hyperplasia, adenomas, intrathyroidal carcinomas, and invasive carcinomas to the thyroid capsule or adjacent tissues[13].

Immunohistochemical staining of thyroid sections

Immunohistochemistry of the thyroid tissue section was performed for the evaluation of the pendrin expression. Specific purified primary polyclonal anti-pendrin antibodies raised in rabbits (ready-to-use) were purchased from Thermo Fisher Scientific (Cat. no. PA5-42060 *SLC26A4*). The localization and expression of pendrin in thyroid follicular cells were done by immunohistochemistry using an indirect method of staining[4] using fluorescent dye. The microscopic

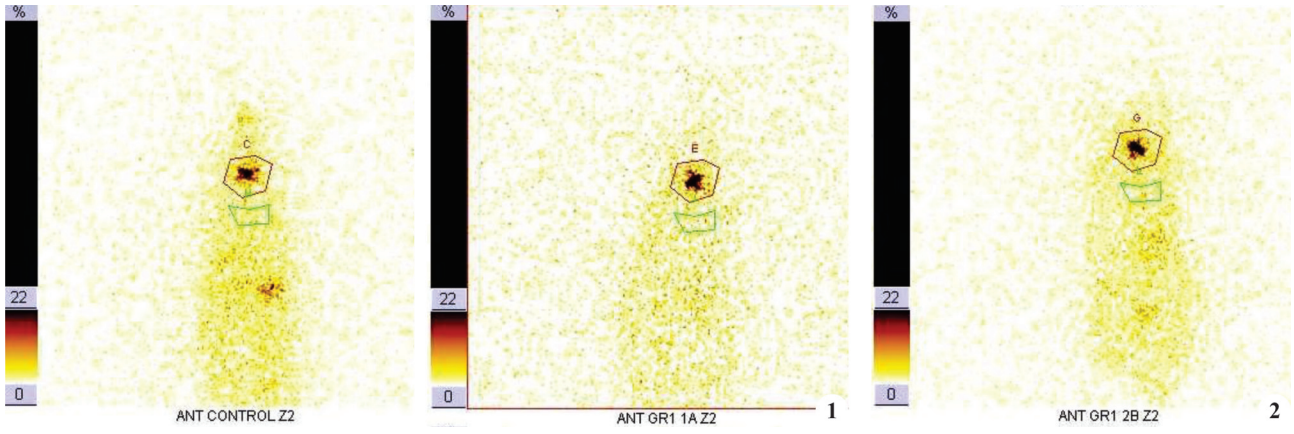

Fig. 1. ^{131}Iodine-scintigraphy scan of the thyroid gland in the control group (Group II) with normal uptake at 24 hours; **Fig. 2.** ^{131}I-scintigraphy scan of the thyroid gland in the DHPN-treated group (Group I) with reduced uptake at 24 hours.

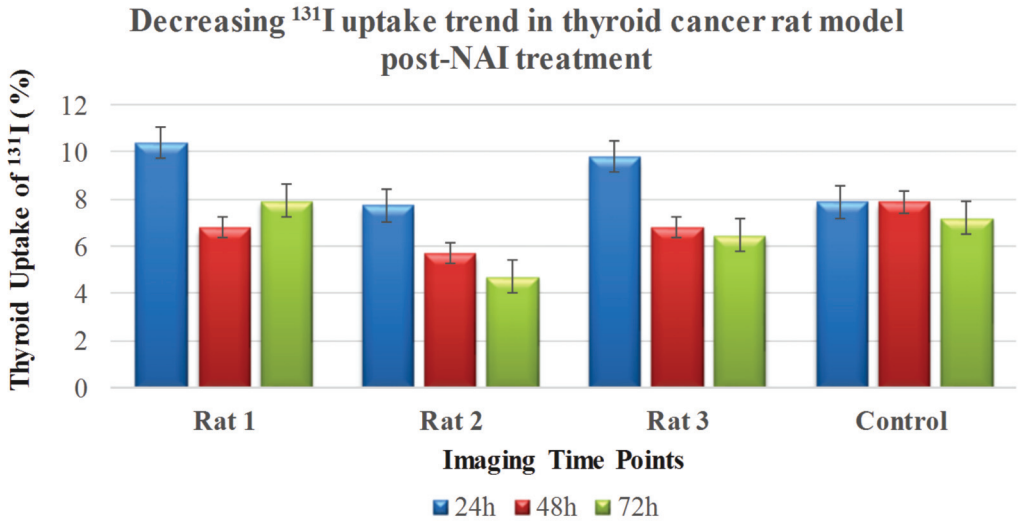

Fig. 3. Decreasing ^{131}I uptake in NAI-treated rats at different time points.

evaluation was performed using a fluorescent microscope (Inverted Leica DMi8 S).

Statistical analysis

As the study was initiated as a pilot experiment, the number of animals was decided and sanctioned by the Institutional Animal Ethics Committee, Bhabha Atomic Research Centre (BARC) for the project. All data are reported as Means ± SD. Student's t-test was used to determine the difference between the treatment group (DHPN) and the control group. The significance level was set at 5% ($p < 0.05$).

RESULTS

Scintigraphy imaging of the thyroid gland

At 24h, ^{131}I uptake by scintigraphy imaging showed thyroid standard uptake values of 13.13±0.115 and 10.16±1.6 in the control (Fig. 1) and DHPN-treated groups respectively (Fig. 2).

However, post-NaI treatment decreasing patterns at 24h, 48h and 72h were observed in a few DHPN-treated animals similar to the control (Fig. 3). Nonetheless, the increasing trend of ^{131}I uptake was also observed in a few DHPN-induced thyroid cancer animals compared to the control group (Fig. 4).

Histopathological observations of thyroid sections

The histological examination of H&E-stained thyroid sections in the healthy control animals showed a normal pattern of follicles of different sizes with colloidal secretion (Fig. 5) in the light microscope. New blood vessel formations and invasion were extended

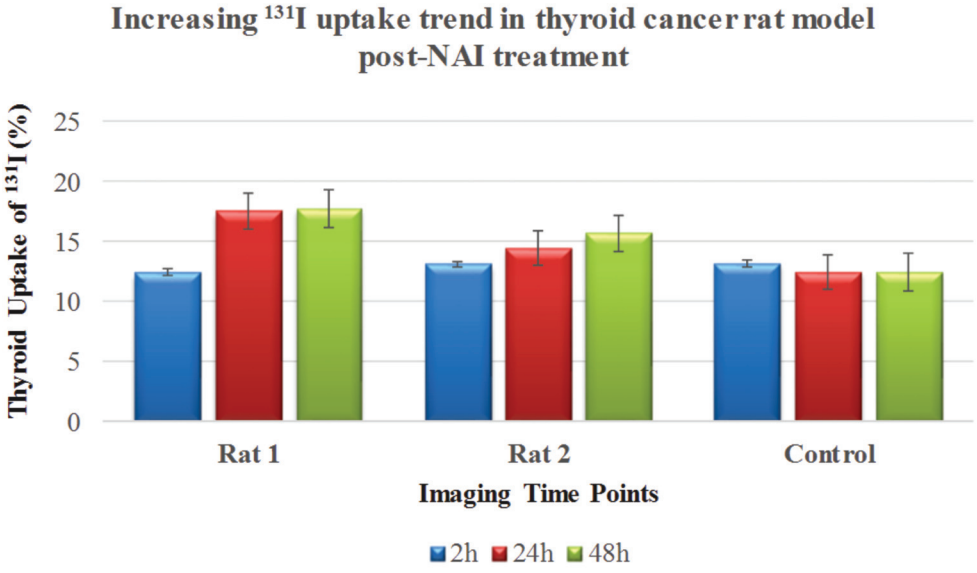

Fig. 4. Increasing ^{131}I uptake in NAI-treated rats at different time points.

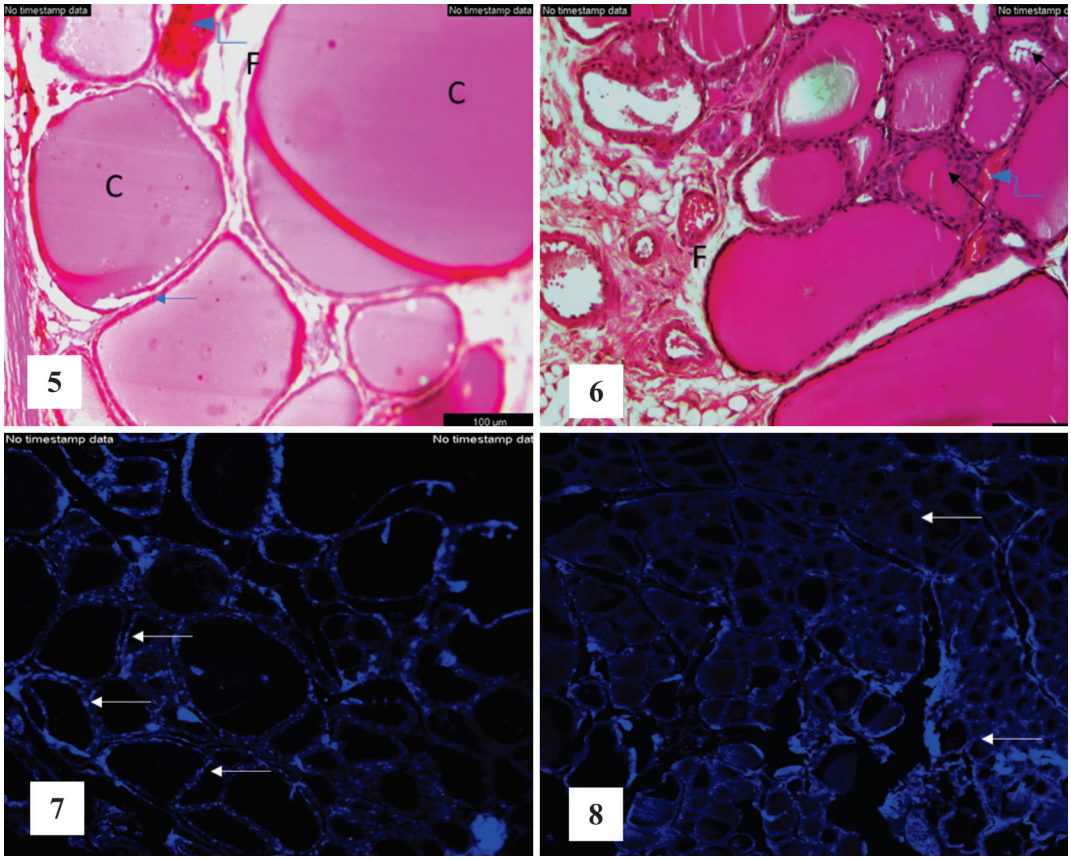

Fig. 5. Representative photomicrograph showing histopathology of the thyrocytes of a male Wistar rat in the control (Group II) with normal follicles (F) with colloidal secretion (C) and lining epithelium (arrow), with H&E (400X). Blood capillaries (zigzag arrow) are seen between thyroid follicles; **Fig. 6.** Representative photomicrograph showing thyrocytes of a male Wistar rat in the DHPN-treated group (Group I) with follicular pattern (arrow) of the thyroid cell (F), stained with H&E (400X). Note the infiltration of follicles by congested capillaries (zigzag arrows); **Fig. 7.** Representative photomicrograph showing immunohistochemical expression of thyrocytes with normal pendrin expression (arrow) at the apical membrane in the control group (Group II) (200X); **Fig. 8.** Representative photomicrograph showing immunohistochemical expression of thyrocytes with reduced pendrin expression (arrow) at the apical membrane in the DHPN-treated group (Group I) (200X).

between thyroid follicles (Fig. 5). Overall, no gross or histopathological abnormalities were recorded in the healthy control. Further, in the DHPN-treated animals, the modifications in the epithelial lining of the thyroid follicles were observed with hyperplastic changes in the multifocal area (Fig. 6) which was observed with lung metastasis (Fig. 9) in one case. The presence of dilated congested blood vessels and congested infiltrating capillaries was noted (Fig. 6).

Immunohistochemical evaluation of thyroid sections

In the control group, immunohistochemical expression of thyrocytes was characterized by normal expression of the protein pendrin at the apical membrane (Fig. 7). On the contrary, in the DHPN-treated animals, the immunohistochemical evaluation showed reduced expression of the pendrin protein at the apical membrane of thyroid follicular cells as compared to the control animals (Fig. 8).

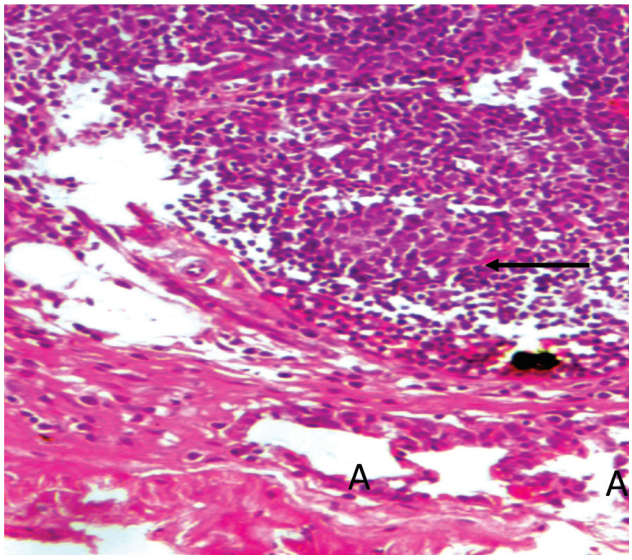

Fig. 9. Representative photomicrograph showing lung metastasis of thyroid cells (arrow) with infiltration of inflammatory cells and adjacent alveoli (A) (200X).

DISCUSSION

The incidence of thyroid cancer has increased over time. The application of gold standard treatment of ^{131}I has been extensively used for the treatment of thyroid cancer disease in humans. However, the pattern of disease has been varying with the patients and the incidence of ^{131}I resistance has risen in about 5-15% of patients with DTC and approximately 50% of metastatic DTCs are refractory to RAI treatment. Iodide uptake and organification are the key features seen in RAI-refractory cancer cells due to the loss of thyroid differentiation features as a result of altered molecular protein signaling pathways that hinder the uptake of ^{131}Iodine in thyroid cancer patients[14]. Several clinical trials with redifferentiating compounds are being carried out for RAI refractivity and the management of RAI-refractive metastatic, recurrent thyroid cancer[5,14]. Considering the overall scenario, we have focused on the expression of pendrin in a chemically induced thyroid cancer model in Wistar rats followed by excess NaI treatment for redifferentiation thereby improving the efficacy of ^{131}I uptake.

Preclinical models have been used for investigating genetic and epigenetic alterations occurring in thyroid neoplasia[5]. Hence, in the present study, animals in experimental group 1 (n=12) were initiated with a single i/p DHPN injection for a period of 3 months whereas the control group (n=6) received saline only. Further, all the animals were put for ^{131}I treatment. The ^{131}I uptake by scintigraphy showed thyroid standard uptake values of 10.16±1.6 and 13.13±0.115 in DHPN-treated and control groups respectively at 24h. The decreased uptake of ^{131}I in thyroid cancer indicated cell proliferation with alteration in cell metabolism that hampered in ^{131}I uptake pattern as compared to control animals.

Further to have more understanding of the reversal effect at the cellular level studies were done with NaI treatment. The NaI is known and tested by Calil-Silveira *et al*., who demonstrated that acute iodide treatment increased *SLC26A4* mRNA content, in both *in vitro* and *in vivo* models[3,15]. Thus, these earlier research findings were considered and extended in our experiments for *in vivo* studies using ^{131}I supported by nuclear imaging techniques to study thyroid uptake in NaI-treated Wistar rats.

Redifferentiation using various epigenetic drugs could imply a valid substitute for targeted therapy in the subgroup of patients with absent iodine uptake by restoring responsiveness to ^{131}I therapy. However, sufficient validation is required for their effectiveness in therapeutic use[5]. In the present study, the experiments were conducted with a single dose of NaI by intraperitoneal route. Post-NaI treatment showed decreasing patterns at 24h, 48h and 72h in a few animals similar to the control. Nonetheless, this could be attributed to an autoregulatory phenomenon known as the 'Wolff-Chaikoff effect' which results from acute iodide excess that transiently impairs thyroid hormone synthesis[15]. These results do not concur with the excess NaI treated in thyroid cancer animals. This could perhaps be due to the inadequate dose of NaI that was planned and used for the redifferentiation purpose. Hence, it is proposed to optimize the dose of NaI for carrying out *in vivo* studies in Wistar rats used as thyroid cancer animal models. Overall, it is observed that the probable reason would be variation in animal species, oxidative stress induced by the chemicals, NaI-induced altered response at the cellular level or changes in thyroid hormone levels. Hence, there are multifactorial processes involved that resolute in the variation in the uptake form seen in animals. However, the rising pattern of ^{131}I uptake was also observed in a few DHPN-induced thyroid cancer animals compared to the control, which could perhaps be related to the effect of iodide excess treatment influencing the expression and functionality of pendrin[15].

The alteration in the thyroid gland was further confirmed with histology indicative of a multifocal area of hyperplastic changes in the epithelial lining of the thyroid follicles which was observed in one case with lung metastasis. Furthermore, the immunohistochemical evaluation showed reduced expression of the pendrin at the apical membrane of thyroid follicular cells in DHPN-treated animals compared to the control animals.

In conclusion, present findings indicate that further research is warranted to verify the role of NaI in the expression of pendrin in conjunction with animal species and age, along with the dose and exposure time of NaI. In addition, the expression and quantification of pendrin at the molecular level could be potentially useful to enhance its utility in the management of RAI refractory human thyroid cancer patients since they represent the main cause of thyroid cancer-related death. Thus, with an optimized dose of NaI, it can serve as a cheap and unique redifferentiation tool in these classes of patients. In this way, redifferentiation would offer a major benefit by avoiding, or at least suspending, long-term systemic treatment in patients who could be readdressed to RAI therapy. Nevertheless, additional studies with sufficient validation are required to confirm the suitability of this approach for therapeutic use in patients.

ACKNOWLEDGEMENTS

The authors are grateful to all the staff members from the animal house facility, RMC, BARC for excellent technical assistance.

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Indian J. Vet. Pathol., 48(1) : 41-49, 2024: DOI: 10.5958/0973-970X.2024.00007.5

RESEARCH ARTICLE

Studies on reproductive pathology and teratogenic effects in experimentally induced hypothyroidism in Sprague Dawley rats and amelioration with *Withania Somnifera* and Shilajit

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Received: 22.8.2023; Accepted: 15.10.2023

ABSTRACT

The experiment was conducted to study the reproductive pathology and teratogenic effects in methimazole induced hypothyroidism in rats and its amelioration. Twenty one days old weaned, female Sprague Dawley rats (96) were divided into 8 groups with 12 animals in each group. They were treated as Group 1: Euthyroid, Group 2: Hypothyroidism induced with methimazole @ 0.02%, Group 3: Hypothyroid + LT4, Group 4: *Withania somnifera* root extract control, Group 5: Hypothyroid + *Withania somnifera* root extract, Group 6: Shilajit control, Group 7: Hypothyroid + Shilajit and Group 8: Hypothyroid + *Withania somnifera* root extract + Shilajit, both @ 100 mg/Kg b. wt. At the end of 3 months, 6 rats from each group were sacrificed. Blood was collected to estimate thyroid profile and estrogen levels. Organs (thyroid, uterus and ovaries) were collected for histopathology studies. The remaining rats were mated with euthyroid adult males and all 48 pregnant rats were administered with treatments as above till the 17th day of gestation. Progesterone levels in pregnant dams and pup survival and mortality rates were estimated. Half of the pups from each group on zero day of their birth were preserved in 10% neutral buffered formalin (NBF) for histopathology (whole pup as a unit) and skeletal staining was done to view the teratogenicity. The remaining half were reared for 21 days and were then sacrificed and various organs (uterus, testis and thyroid) were collected for histopathology studies. The sections of the thyroid gland, reproductive organs (parent stock and F_1 generation) and visceral organs of the whole pup of group II rats revealed marked degenerative changes. Pups stained with Alizarin red S from group II showed skeletal deformities. The present study showed teratogenicity and deviations in reproductive organs in the hypothyroidism model and the usage of herbal drugs have shown improvement in alleviating the toxic effects of hypothyroidism.

Keywords: Histopathology, hypothyroidism, methimazole, shilajit, teratogenicity, *Withania somnifera*

INTRODUCTION

Thyroid hormones are metabolic hormones produced by the thyroid gland and are present in two main forms: the prohormone thyroxine (T_4) and the biologically active form triiodothyronine (T_3). THs play a crucial role in various aspects of an individual's life, for example, development, metabolism and reproduction, across vertebrates, including humans[1]. Maternal hypothyroidism (MH) is the most common cause of transient congenital hypothyroidism. Regarding development of fetal body systems during pregnancy, interference at different times provides different results and the appropriate time for induction of hypothyroidism should be selected based on accurate time of development of the system under assessment[2]. In females hypothyroidism can fabricate reproductive problems and results in improper progress of offspring with stunted growth and mental retardation if untreated[3].

Scientific investigations towards the mitigation of thyroid disorders by the plant extracts are running fast but, the solitary hitch was that, only one thyroid hormone among T_3, T_4 or TSH was altered by the plant extract[4]. Though many herbs are available for the effective function of thyroid hormones, the present study was conducted to find out the efficacy of the herbs that can regulate the levels of thyroid hormones independently or in combination by using *Shilajith and Withania*

How to cite this article : Ramya, B., Kumar, A.A., Reddy, A.G., Purushotham, G and Shivakumar, P. 2024. Studies on reproductive pathology and teratogenic effects in experimentally induced hypothyroidism in Sprague Dawley rats and amelioration with *Withania Somnifera* and Shilajit. Indian J. Vet. Pathol., 48(1) : 41-49.

Table 1. Serum thyroid profile in different groups of rats (Parent stock).

Groups (n=6)	T_3 (ng/dl) concentration	T_4 (µg/dl) concentration	TSH (µ IU/ml) concentration
Group 1	115.68±4.46^b	4.71±0.14^b	0.05±0.01^a
Group 2	101.15±4.06^a	3.01±0.24^a	5.91±0.45^c
Group 3	110.73±4.84^b	4.36±0.18^b	0.87±0.03^b
Group 4	118.03±4.69^b	4.79±0.17^b	0.03±0.007^a
Group 5	111.74±4.17^b	4.24±0.17^b	1.04±0.03^b
Group 6	116.62±5.02^b	4.69±0.19^b	0.04±0.009^a
Group 7	106.78±4.71^a	3.45±0.12^a	1.91±0.16^b
Group 8	113.19±4.76^b	4.31±0.21^b	0.51±0.12^b

Means with different superscripts differ significantly ($p<0.05$), One way ANOVA (SPSS:15)

somnifera.

MATERIALS AND METHODS

Ninty six female rats (21 days old weaned) *Sprague-drawly* rats were picked up from Mahaveera Enterprises, Hyderabad. Rats were nursed in the modern animal house of the Department of Veterinary Pharmacology and Toxicology with a 12 h - 12 h dark and light cycle at a temperature of 22-24^{O}C. Rats were enclosed in hard base polypropylene cages, placed on standard pellet feed and provided *ad libitum* water. Rats were adapted for five days before the experiment. The study was conducted with prior approval of the Institutional Animal Ethics Committee, CVSc, Rajendranagar (I/6/16/05-01-16).

Rats were distributed into 8 groups with 12 in each group and the experiment was conducted for 3 months as per the technical program given below:

Group 1: Euthyroid

Group 2: Hypothyroidism induced with Methimazole @ 0.02%

Group 3: Hypothyroid + Levothyroxine

Group 4: *Withania somnifera* root extract control @ 100 mg/Kg b. wt.

Group 5: Hypothyroid + *Withania somnifera* root extract @ 100 mg/Kg b. wt.

Group 6: *Shilajit* control @ 100 mg/Kg b. wt.

Group 7: Hypothyroid + *Shilajit* @ 100 mg/Kg b. wt.

Group 8: Hypothyroid + *Withania somnifera* root extract @ 100 mg/Kg b. wt. + *Shilajit* @ 100 mg/Kg b. wt.

Thyroid profile and estrogen levels were estimated from sera of blood collected at the end of the third month by Radio Immuno assay employing DiaSorin S.p.A. kits, USA. At the end of 3 months, 6 rats from each group were sacrificed and various organs (uterus, ovary and thyroid) were collected in 10% neutral buffered formal saline for histopathological studies.

Experiment

Left over 48 female rats (6 from each group) from experiment 1 were mated with 24 euthyroid adult (above 3 months) males (from Mahaveera Enterprises, Hyderabad) and all the 48 pregnant rats were administered with treatments as in experiment 1, up to 17th day of gestation.

Progesterone levels in pregnant dams were estimated in the collected serum samples by Radio Immuno assay employing DiaSorin S.p.A. kits, USA. Pups from all the groups were screened for survival and mortality rates. Half the number of the pups from each group on the day of their birth was preserved in 10% neutral buffered formalin (NBF). A few of the preserved pups were processed for histopathology[5], to study the section of whole pup as a unit and a few pups were subjected to skeletal staining with alizarin red-S[6] to view the teratogenicity. The remaining half balance pups were reared till 21 days. The rats were then sacrificed and various organs (uterus, testis and thyroid) were collected in suitable preservatives for histopathology studies.

RESULTS

Thyroid Profile

The serum T_3 and T_4 hormone levels (ng/dl) of group 2 were significantly ($p<0.05$) lesser than group 1 while the values in groups 3, 4, 5, 6 and 8 were analogous to group 1. T_3 and T_4 concentration in group 7 was significantly ($p<0.05$) inferior to that of group 1. The serum TSH hormone concentration (µ IU/ml) of group 2 was significantly ($p<0.05$) elevated than group 1 and was significantly lesser in groups 3 to 8 (Table 1).

Serum Estradiol (pg/ml)

The serum estradiol concentration (pg/ml) of rats in group 2, was significantly ($p<0.05$) elevated than control groups and treatment groups (Table 2).

Serum Progesterone (ng/ml)

The serum progesterone concentration (ng/ml) in groups 1, 3, 4 and 5 was lower than in groups 2, 6 and 7, while it was significantly ($p<0.05$) higher in group 8 (Table 2).

Table 2. Serum estradiol (pg/ml) and progesterone concentration (ng/ml) concentration in different groups of rats (Parent stock).

Groups (n=6)	Estradiol concentration (pg/ml)	Progesterone concentration (ng/ml)
Group 1	23.72±3.07^{a}	47.26±3.19^{a}
Group 2	37.43±3.78^{c}	57.67±5.46^{ab}
Group 3	29.57±3.23^{b}	54.12±4.12^{a}
Group 4	22.94±2.79^{a}	53.71±4.14^{a}
Group 5	31.66±3.07^{b}	49.22±3.67^{a}
Group 6	21.03±3.15^{a}	59.43±5.44^{ab}
Group 7	33.07±4.01^{b}	56.04±5.01^{ab}
Group 8	26.35±2.87^{ab}	61.72±5.01^{b}

Means with different superscripts differ significantly ($p<0.05$), One way ANOVA (SPSS:15)

Pup survival/mortality

Mortality was not observed in pups and the survival rate was maintained till weaning in all the groups.

In parent stock very mild reduction in the size of thyroid in group II rats and no appreciable or significant gross or histological lesions were observed in any of the experimental groups.

Thyroid gland

The section of thyroid in group II, showed vacuolation, a few follicles contained varied amounts of colloid and the others were devoid of colloid (Fig. 1). In groups V and VII the alterations were mild, nearly comparable with that of control groups (Fig. 2) and in group VIII, the changes are comparable with that of control groups.

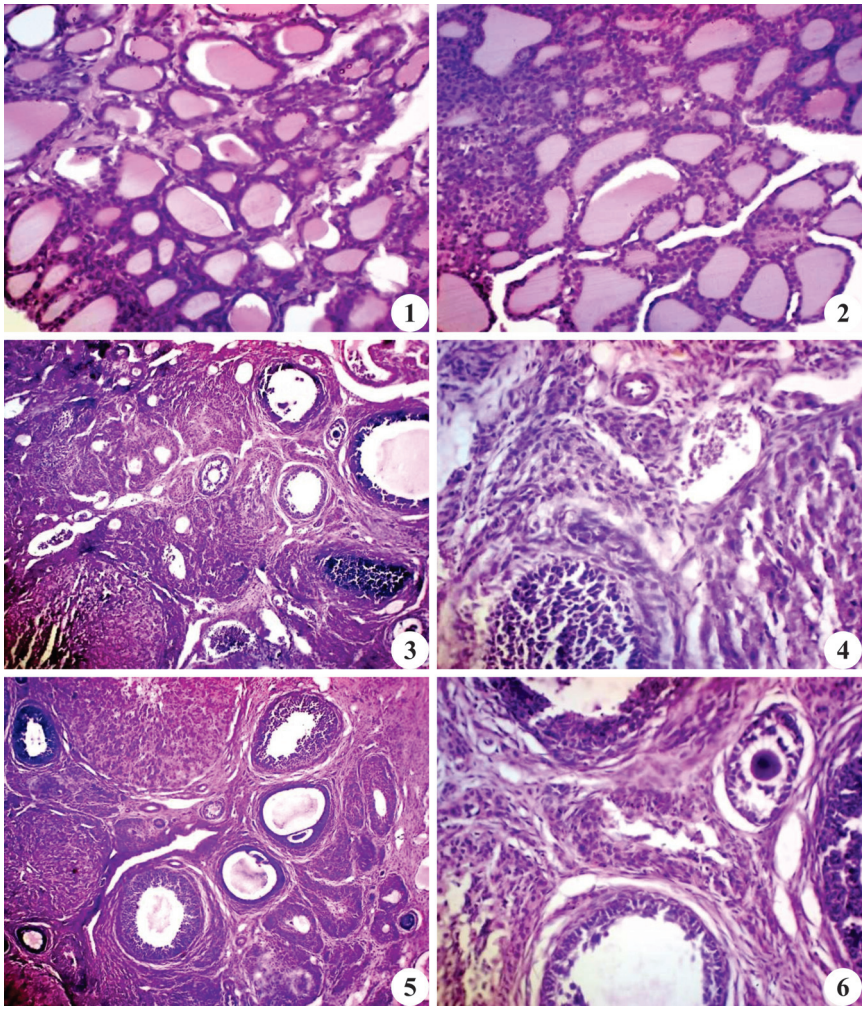

Fig. 1. Section of thyroid gland showing vacuolation, some follicles contained varied amounts of colloid, the others were devoid of collod in group II (H&E X400); **Fig. 2.** Section of thyroid gland showing mild changes in follicles with varied amounts of colloid in group VII (H&E X400); **Fig. 3.** Section of ovary showing moderately degenerated tertiary follicle mainly along with primordial, primary and secondary follicles. Increase in thickness of tunica albuginea, atretic/cystic spaces, oedema, vacuolar degeneration, degenerated oocyst in tertiary follicle in group II (H&E X400); **Fig. 4.** Section of ovary showing fibroblasts proliferation with thickened endothelium in group II (H&E X400); **Fig. 5.** Section of ovary showing mild oedema and degeneration of follicles in group V (H&E X400); **Fig. 6.** Section of ovary showing mild degenerations in follicular granulosa and follicles in group VII (H&E X400).

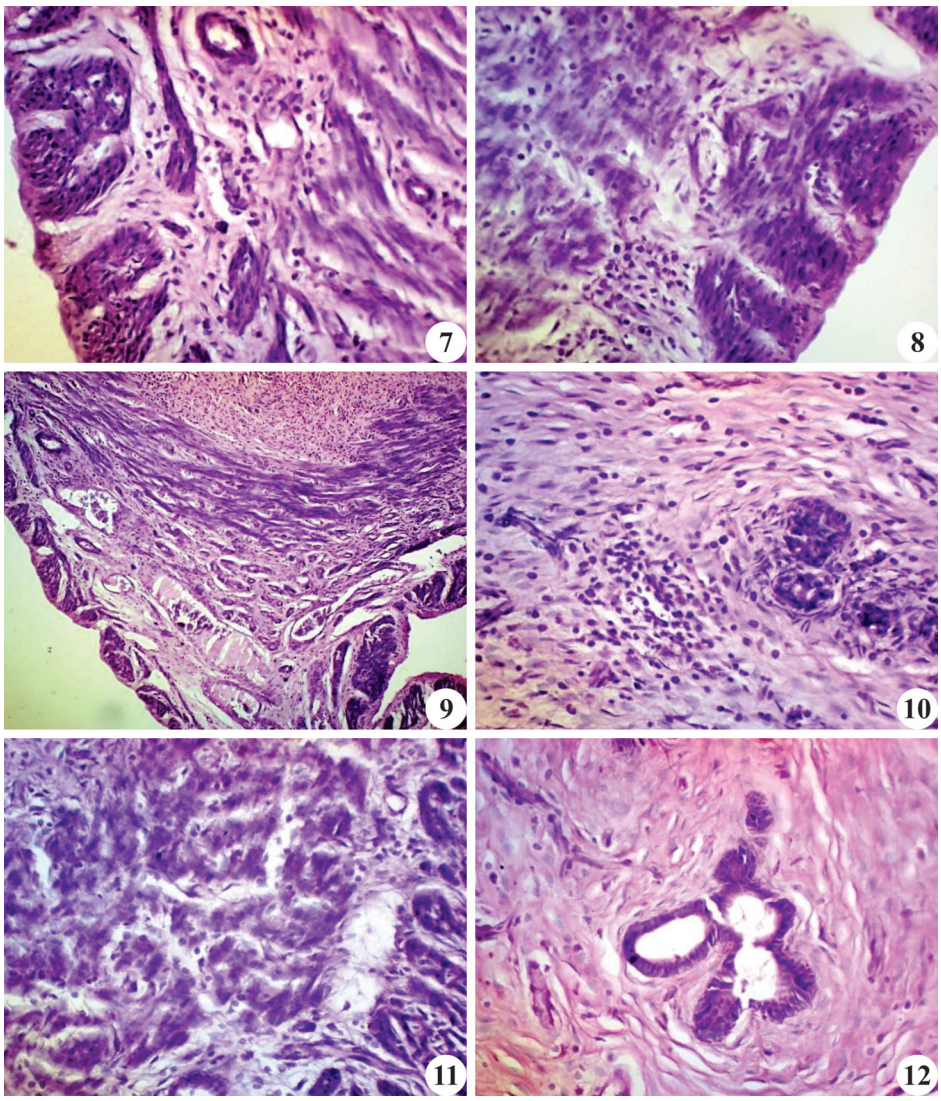

Fig. 7. Section of uterus showing glandular hyperplasia and fibroblasts proliferation in group II (H&E X400); **Fig. 8.** Section of uterus showing mono nuclear cell infiltration in group II (H&E X400); **Fig. 9.** Section of uterus showing thickened endometrium, disruption, oedema, congestion and hemorrhages in group II (H&E X100); **Fig. 10.** Section of uterus showing severe mononuclear cell infiltration and lymphoid aggregates in group II (H&E X400); **Fig. 11.** Section of uterus showing glandular hyperplasia in endometrium in group II (H&E X400); **Fig. 12.** Section of uterus showing thick luminal epithelium and fibroblasts proliferation in group II (H&E X400).

Ovary

In group II, moderately degenerated tertiary follicles mainly along with primordial, primary and secondary follicles, increase in thickness of tunica albuginea, atretic/cystic spaces, oedema, vacuolar degeneration, degenerated oocyst in tertiary follicle were seen (Figs. 3 and 4). In group V and VII, the changes were mild (Figs. 5 and 6) and in group VIII, the changes were almost comparable to normal cosntrol groups.

Uterus

In group II, glandular hyperplasia and fibroblasts proliferation, mono nuclear cell infiltration, thickened endometrium, disruption, oedema, congestion and hemorrhages were noted (Figs. 7-12). In groups V and VII, discontinuity in surface epithelium with mild to moderate congestion and hyalinization in the circular muscle layer of myometrium was noted (Fig. 13). In group VIII, the surface epithelium was thrown into folds and other changes were comparable to that of normal control groups.

Histopathology of whole pup as a unit

In group II, the section of the liver showed fatty change, an increase in sinusoidal spaces with mild degenerative changes, central vein congestion and mononuclear cell infiltration (Figs. 14 and 15) and the kidney showed degenerative changes in developing tubules and glomeruli (Fig. 16). In groups V and VII, mild degenerative changes were noted in the brain (Fig. 17).

In F_1 Generation**:** No appreciable or significant

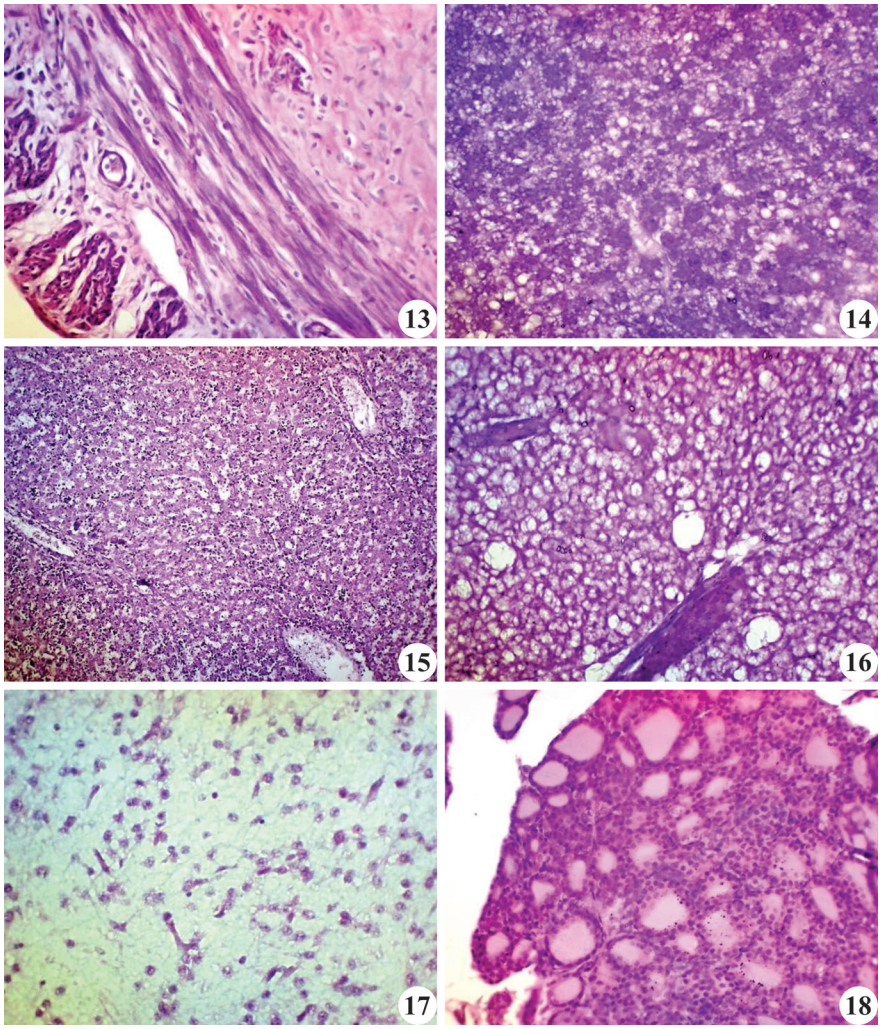

Fig. 13. Section of uterus showing discontinuity in surface epithelium with mild to moderate congestion and hyalinisation in circular muscles in group VII (H&E X400); **Fig. 14.** Section of liver showing fatty change, increase in sinusoidal spaces with mild degenerative changes in group II (Whole pup) (H&E X400); **Fig. 15.** Section of liver showing central vein congestion and mononuclear cell infiltration in group II (Whole pup) (H&E X400); **Fig. 16.** Section of kidney showing fatty change, degenerative changes with developing tubules and glomeruli in group II (Whole pup) (H&E X400); **Fig. 17.** Section of brain showing mild degenerative changes in group VII (Whole pup) (H&E X400); **Fig. 18.** Section of thyroid gland showing altered follicles with variation in shape and size, increase number of C cells focally and increase in interfollicular space in group II (H&E X400).

gross or histological lesions were observed in any of the experimental groups.

Thyroid gland

The section of thyroid in group II showed altered follicles with variation in shape and size, an increased number of C cells focally and an increase in interfollicular space (Fig. 18) and in group VIII the changes were almost comparable with that of normal control groups.

Testis

In group II, semniferous tubules degeneration, Leydig cells accumulation in the center, disruption of basement membrane along with tubular epithelium and cuboidal cells at the center were prominent. Different stages of spermatozoa with few defects and moderate hyperplasia were noted (Figs. 19 and 20). In groups V and VII, mild to moderate hyperplasia and hypertrophied semniferous tubules with mild changes in the basement membrane were noted. In group VIII the changes were almost comparable with that of normal control group (Fig. 21).

Uterus

In group II, vacuolation and hyperplasia of glands were recorded (Fig. 22). In groups V and VII, discontinuity in surface epithelium, mild to moderate hyalinisation in the circular muscle layer of myometrium and mild to moderate hyperplasia was noted. In group VIII, the changes were comparable to that of normal control groups (Fig. 23).

Teratogenecity

Skeletal staining with Alizarin red S was performed

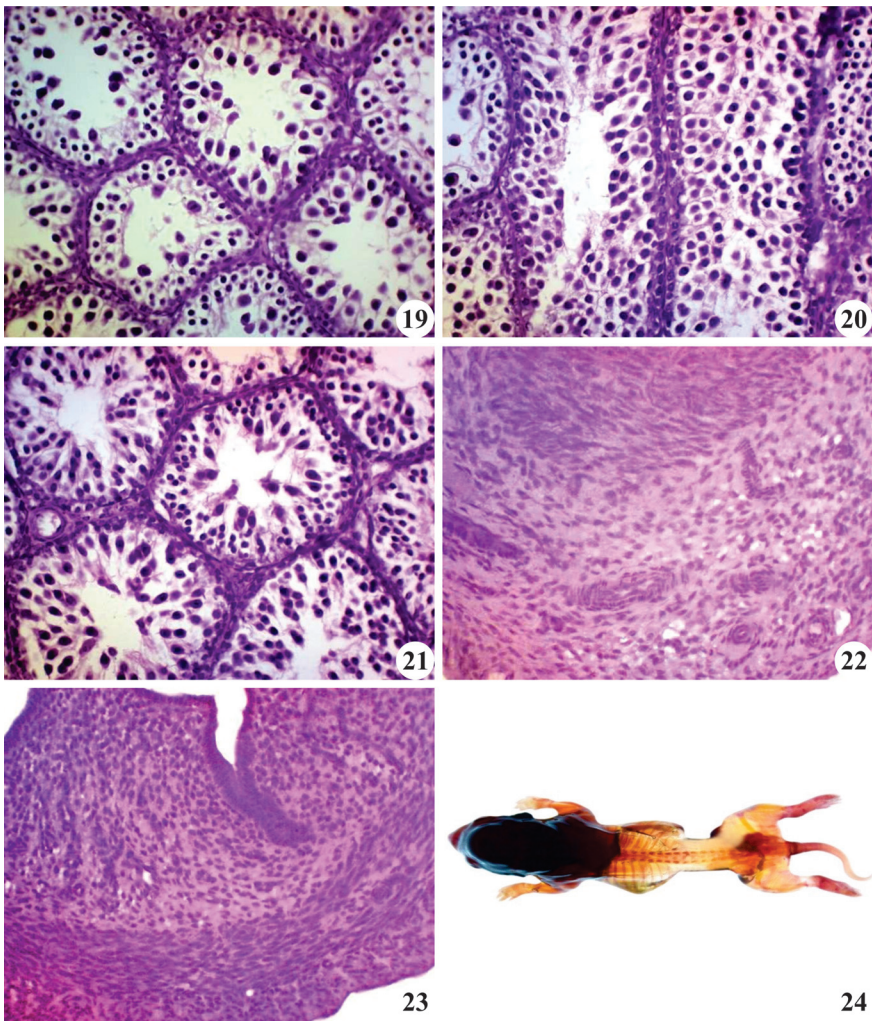

Fig. 19. Section of testis showing different stages of spermatozoa with few defects in group II (H&E X400); **Fig. 20.** Section of testis showing semniferous tubular degenerations, accumulated ley dig cells in the centre and disruption of basement membrane along with tubular epithelium in group II (H&E X400); **Fig. 21.** Section of testis almost comparable with that of normal control groups in group VIII (H&E X400); **Fig. 22.** Section of uterus showing vacuolation and hyperplasia of glands in group II (H&E X400); **Fig. 23.** Section of uterus showing no changes and can be comparable to that of normal control groups in group VIII (H&E X400); **Fig. 24.** Pup showing moderate lateral deviation at distal half of spine with under developed vertebrae, thin ribs, loss of growth plate, improper bone alignment in hind limbs in group II.

to pups belonging to all groups. The pups from group II, showed skeletal deformities like reduction/absence of growth plate thickness, bending of bones like femur, hidden humerus, lateral deviation of distal half of spine, variation in inter vertebral space with undeveloped/ underdeveloped vertebrae, deviation in collateral alignment of ribs, increased intercostals spaces and thinning of ribs (Figs. 24 and 25). Pups from group V, showed normal growth plates with mild lateral deviation at the distal part of spine (Fig. 26). Pups from group VII showed improved intercostal space with developed growth plates and mild widening of proximal half of the vertebral column (Fig. 27). Pups from group VIII showed well developed growth plates improved intercostal space (Fig. 28).

DISCUSSION

Thyroid profile

The significant ($p<0.05$) decrease in serum T_3 and T_4 hormone concentration (ng/dl) and significant ($p<0.05$) increase in TSH hormone concentration (μ IU/ml) of the group 2 might be due to methimazole's inhibition of the enzyme thyroperoxidase, that acted in thyroid hormone synthesis by oxidizing the anion iodide (I^-) to iodine (I_2), hypoiodous acid (HOI), enzyme linked hypoiodate (EOI) facilitating iodine's addition to tyrosine residues on the hormone precursor thyroglobulin, a necessary step in the synthesis of triiodothyronine (T_3) and thyroxine (T_4). The decrease in T_3 and T_4 through negative feedback mechanism might have stimulated pituitary to release more thyroid stimulating hormone[7].

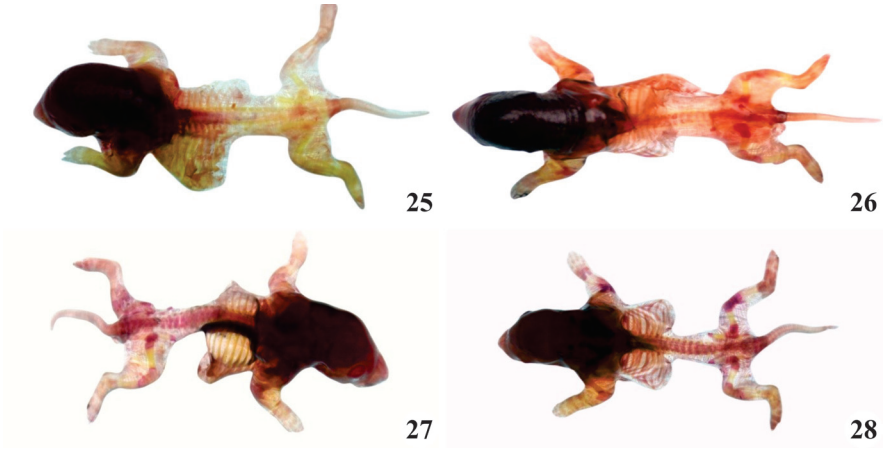

Fig. 25. Pup with hidden humerus in right fore limb, bending of femur in left hind limb and broad paw in right hind limb in group II; **Fig. 26.** Pup showing normal growth plates with mild lateral deviation at the distal part of spine in group V; **Fig. 27.** Pup with improved intercostal space with developed growth plates and mild widening of proximal half of the vertebral column in group VII; **Fig. 28.** Pup with well developed growth plates improved intercostal space in group VIII.

Increase in thyroid hormone synthesis in group 5 rats might be due to immunomodulation effect of herb that has a stimulatory effect on a sluggish thyroid and increased serum T_4 concentration[8].

Shilajit improved thyroid profile by controlling thyroid gland function through it's adaptogenic activity[9].

Estrogen and Progesterone

The significant ($p<0.05$) increase in serum estradiol concentration (pg/ml) in hypothyroid group might be due to the result of a permissive action of prolactin that augments the number of luteal LH receptors which favours gonadotrophic action that in turn promotes the conversion of progesterone into androstenodione and then to estradiol[10]. Further, increased circulating estradiol stimulates expression of cyp19A1 aromatase that binds elevated estrogen receptor beta (ERβ) in granulosa and luteal cells that in turn stimulates estradiol synthesis and release. Thus, a positive feedback loop might have established and lead to a sustained increase in circulating estradiol during hypothyroidism[11].

The serum progesterone concentration (ng/ml) in group 2 on days 15-21 of pregnancy was higher than other groups might be due to increased prolactin concentrations in altered thyroid profile[3].

The effect of the ashwagandha and *shilajit* on gonadal hormone levels might be due to antioxidant effect of herbs which as a reproductive function probably was performed via inhibition of oxidative stress. These can also be correlated with the histopathological studies of the reproductive organs of the present study[8].

Pup survival/mortality

Mortality was not observed in pups and survival rate was maintained till weaning in all the groups might be due to absence of iodine deficiency and presence of only thyroid hormone deficiency which alone cannot result mortality in pups[12].

Thyroid Gland

The lesions observed in the section of thyroid gland in group II of both parent and F_1 generation were similar with degree of variation. Follicles shape and size, amount of colloid varied. Some follicles were devoid of colloid Conversion of cell type in the follicular epithelium with presence of C cells and para follicular cells. The changes observed might be due to functional induction of methimazole on thyroid gland[13]. In groups V and VII, improvement in the structure of follicles and their function was observed and in group VIII, no lesions were noted. These findings might be due to improved functioning of thyroid gland by stimulating the thyroid stimulating hormone by action of *Withania somnifera*[14].

Uterus, Ovary and Testis

In group II the sections of the reproductive organs in parent stock as well as F_1 generation revealed almost in similar nature with degree of variation. In uterus congestion, hemorrhages in endometrium, hyperplasia of endometrial and myometrial glands, mononuclear cell infiltration, fibroblasts proliferation and hyalinization were noted[15].

In ovary (parent stock) interfollicular thickening, edema, thickened endothelium, degenerated primordial, primary, secondary and tertiary follicles[16], degenerated oocysts were recorded[17].

In testis (F_1 generation), semniferous tubule degeneration, leydig cells accumulation towards centre and degenerated cells[16], disruption of basement membrane and various stages of defective spermatozoa were observed[18].

These findings might be due to inhibitory action of

thyroid hormones in turn on estrogen and progesterone secretions that can be correlated with the results of present study. The improved lesions in group V and VII and almost no lesions in group VIII might be due to improved functioning of endocrine glands in turn maintenance of balance of hormonal secretion by the action of *Withania somnifera*, *shilajit* and their combination[19,20].

Whole pup

The histopathological lesions in various organs processed as whole pup in group II revealed neurofibrillary degeneration in brain, mild degeneration and increase in interfibrillary spaces with developing myocytes in heart, altered follicles and presence of colloid in few follicles in thyroid gland, severe fatty change, increased sinusoidal space with mild degenerative changes, mononuclear cell infiltration in liver and fatty change, degenerative changes with developing tubules and glomeruli in kidney. The changes observed might be due to structural and functional inhibition of immune organs as well as others that might have acquired from parent stock that were treated with methimazole for inducing hypothyroidism and further the thyroid gland functioning initiates from 15th day onwards in fetus[21]. So this can be attributed as up to first two weeks of fetal stages the changes in the organs must be due to inheritance and further the improvement might be due to function of its own thyroid gland. No literature on this aspect was available and may be the first report.

The improved status in groups V and VII and in group VIII can also be explained as above with relevancy.

Teratogenisity

Pups from group 2 showed skeletal deformities like reduction/absence of growth plate thickness, bending of bones like femur, hidden humerus, lateral deviation of distal half of spine, variation in inter vertebral space with undeveloped/underdeveloped vertebrae, deviation in collateral alignment of rib 6, increased intercostal spaces and thinning of ribs. These abnormalities might be due to administration of methimazole that might have reulted developmental hypoplasia and functional hypothyroidism, leading to severe embryopathies in fetuses[22]. Skeletal changes in pups might be due to inhibition of GSH synthesis or reduced GSH, that might act as detoxificant of a xenobiotic free radical intermediate and protects embryo from tereatogenic effects. GSH might also act as cytoprotective against oxidative stress[23].

Pups from rats of groups 5 and 7 revealed less teratogenic effects compared to group 2 due to antioxidant actions of W*ithania somnifera* and *shilajit* along with free radical scavenging nature, thus reduced free radicals decrease GSH which was a major terarogenic agent[8,24].

In the present study, amelioration effect of *Withania somnifera* was more than *Shilajit* might be due to its slow action on the injured cell and the combination of both drugs had synergystic effect resulted in high amelioration than *Withania* alone as ayurvedic medicine generally prefers combinations of herbs rather than single herbs[25].

CONCLUSION

Hormonal and histopathological alterations in the present study indicated reproductive organ damage at the molecular level. Whole pup histopathology indicated organ damage might be due to induced hypothyroidism. The skeletal abnormalities can be well attributed for induced hypothyroidism. Ameliorating actions of *Shilajit* alone were less significant than *Withania somnifera* alone and combination of the herbs. Administration of *Withania somnifera* and *Shilajit* individually and combined alleviated the deleterious effects caused by hypothyroidism due to their antioxidant and endocrine stimulant properties. Synergistic action of herbs in ameliorating the effects of hypothyroidism is far better than administration of individual herbs.

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Indian J. Vet. Pathol., 48(1) : 50-61, 2024: DOI: 10.5958/0973-970X.2024.00008.7

RESEARCH ARTICLE

Studies on pancreatic pathology of poultry in different diseases in relation to season, age, sex and varieties of birds

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Received: 8.8.2023; Accepted: 4.10.2023

ABSTRACT

Poultry have a well-developed pancreas which is in the abdominal cavity in between the loop of duodenum. Present study was taken up to evaluate the pancreatic pathology of poultry in different diseases in relation to season, age, sex, and varieties of birds. The study spanned over a period of one year from December 2017 to November 2018. Pancreatic pathology was assessed at the time of performing postmortem examination of all the birds submitted to the Department of Veterinary Pathology, College of Veterinary Science & Animal Husbandry (C.V.Sc. &AH), Ranchi, Jharkhand. In our study, 22.23% birds showed definite pancreatic pathology in eight different varieties of birds. Kadaknath birds were most susceptible (85.71%) followed by broiler birds (75.34%), whereas, Punjab breed semi synthetic line (PB2) variety, Crossbreed, Jharsim and Desi birds showed moderate susceptibility, while Delham Red (DR) and Vanraja variety showed lesser susceptibility. Season wise study showed highest percentage of pancreatic pathology in monsoon (28.84%) followed by summer (22.57%) and least in winter (12.92%) season. A significant variation was observed in the susceptibility to pancreatic pathology under different disease conditions between different age groups of birds. Age wise highest pancreatic pathology was registered in grower birds (33.69%) followed by chicks (20.06%) and adult birds (19.54%). The incidence of pancreatic pathology was significantly higher in female (52.49%) than male (47.51%) birds.

Keywords: Age, pancreatic pathology, poultry, season, sex

INTRODUCTION

The pancreas is a vital part of gastrointestinal system and endocrine system for digestive function and glucose metabolism respectively. Poultry and other birds have a well-developed pancreas, which is located between the loops of duodenum. It is relatively small in carnivore and granivore birds but larger in piscivore and insectivore birds.

Diseases of the pancreas can affect the exocrine pancreas, endocrine pancreas, or both[1]. Pathomorphological studies of pancreas in avian diseases has been reported by Kumar *et. al* (2021). Pancreatic pathology can also develop due to non-infectious causes such as zinc toxicosis[3,4,5] or copper[6] and selenium[7] deficiency.

The pancreatic morphology also shows age related variations. Age related involution have been described in pancreas[8]. In senile conditions, pancreas undergoes atrophic changes and atrophy of acinar cells decreases the functional status of pancreas. Nature of diet has also been reported to alter the proportion of different enzymes in pancreatic secretion whereby the secretion of enzymes specific for particular diet shows enhancement. The highest percentage of enzyme in pancreatic secretion is made of procaroxypeptidase A and B (29.8%), followed by amylase (28.9%), chymotrypsin A, B and C (20%), trypsin inhibitor (11.3%) and trypsinogen (10%) (Pubols, 1993). The age related and diet related variation under the influence of different seasons and in different varieties of birds make poultry susceptible to physiological alterations, consequently having effect on their performance.

The incidence of various infectious diseases shows distinct age susceptibility and seasonal variations. Apart from characteristic pathology observed due to infectious, nutritional, toxic, or metabolic diseases; significant population of birds show poor body weight gain or run-down condition of idiopathic nature. In such birds, pancreatic dysfunction may constitute an important underlying factor for poor digestibility of carbohydrate, fat and protein or hormonal imbalance between glucagon, insulin and somatostatin with subsequent loss of body weight. Present study was taken up to evaluate the pancreatic pathology of poultry in different diseases in relation to season, age, sex, and varieties of birds.

How to cite this article : Kumar, B., Gupta, M.K., Kumar, S. and Lakra, P.P. 2024. Studies on pancreatic pathology of poultry in different diseases in relation to season, age, sex and varieties of birds. Indian J. Vet. Pathol., 48(1) : 50-61.

MATERIALS AND METHODS

Pancreatic pathology was

assessed during postmortem (PM) examination of all the birds submitted to the Department of Veterinary Pathology, (CVSc & AH), Ranchi. Necropsy was carried out as per approved procedure described by Chauhan and Roy[9]. To avoid putrefaction, PM examination was conducted twice daily in the morning and evening. Pancreas and other organs were examined critically for the presence of gross lesions. For histopathological examination, pancreatic tissue was collected from the areas where gross changes were most distinct. The collected tissues were routinely processed for preparation of 4-5 µ thick pancreatic section and stained with Ehrlich's haematoxylin and eosin stain[10]. Histochemical examination for detection of fibrous connective tissue was also carried out by Mallory's Trichrome method (Crooke-Russell Modification)[11]. Preliminary diagnosis of the disease affecting the birds was made on the basis of gross pathological changes which were systematically recorded. Wherever required during the study, bacteriological, serological, or pathological examination was carried out for confirmation of the disease conditions.

Diagnosis of bacterial diseases such as colibacillosis, pasteurellosis was done on the basis of characteristic gross lesions followed by identification of the organisms on the basis of colony characteristics, staining property and biochemical characteristics after their isolation on bacteriological media such as nutrient agar, MacConkey agar or blood agar. Viral diseases such as infectious bursal disease (IBD), infectious bronchitis (IB) and ranikhet disease (RD) were diagnosed on the basis of classical gross lesion and serologically by ELISA tests, whereas diagnosis of fowl pox, lymphoid leucosis, cystadenocarcinoma and chicken infectious anaemia was based on characteristic gross pathology.

Diagnosis of brooders pneumonia was done on the basis of characteristic gross lesions and demonstration of fungal hyphae in a wet preparation stained with lactophenol cotton blue stain. Mycotoxicosis was confirmed by

Table 1. Incidence of pancreatic pathology in different disease conditions in decreasing order of frequency within individual group of disease along with incidence of gross pancreatic lesions in different disease conditions of poultry.

Types of Diseases	Disease	No. of Birds affected	Pancreas Affected	% of Pancreas affected	Bleached	Congestion	Mottled with Necrotic Foci	Deformed Pancreas	Atrophied	Hyperplastic
Viral	I.B.D.	19.00	18.00	94.74	55.56	22.22	11.11	50.00	5.56	0.00
	I.B.	15.00	14.00	93.33	21.43	50.00	14.29	35.71	0.00	0.00
	Cystadeno carcinoma	4.00	3.00	75.00	0.00	0.00	0.00	0.00	100.00	0.00
	Fowl Pox	4.00	3.00	75.00	66.67	33.33	33.33	0.00	33.33	0.00
	Lymphoid Leucosis	34.00	20.00	58.82	35.00	20.00	55.00	5.00	5.00	5.00
	Chicken Infectious anaemia	42.00	21.00	50.00	14.29	71.43	14.29	28.57	0.00	4.76
	R.D.	2124.00	289.00	13.61	39.10	33.22	49.83	7.27	2.08	2.42
Bacterial	Pasteurellosis	21.00	19.00	90.48	26.32	47.37	36.84	0.00	0.00	0.00
	Oophoritis	38.00	23.00	60.53	26.09	34.78	43.48	0.00	0.00	0.00
	Coryza	55.00	24.00	43.64	16.67	54.17	37.50	25.00	4.17	2.30
	Enteritis	177.00	77.00	43.50	32.47	41.56	7.79	15.58	2.60	3.90
	Collibacillosis	47.00	15.00	31.91	33.33	46.67	46.67	53.33	0.00	0.00
	Yolk Sac Infection	329.00	87.00	26.44	36.78	48.28	9.20	5.75	1.15	0.00
	Pneumonia	368.00	61.00	16.58	29.51	63.93	16.39	11.48	1.64	4.17
Fungal	Brooder Pneumonia	6.00	5.00	83.33	40.00	20.00	40.00	40.00	0.00	0.00
Parasitic	Ascaridiasis	21.00	6.00	28.57	66.67	0.00	50.00	0.00	0.00	0.00
Protozoal	Coccidiosis	138.00	29.00	21.01	82.76	6.90	10.34	10.34	10.34	3.45
Metabolic	Chick Oedema Disease	26.00	23.00	88.46	8.70	60.87	17.39	56.52	0.00	1.64
	Gout	9.00	6.00	66.67	16.67	16.67	66.67	0.00	0.00	0.00
Toxicological	Aflatoxicosis	27.00	22.00	81.48	18.18	27.27	31.82	27.27	0.00	9.09
	Ochratoxicosis	292.00	79.00	27.05	53.16	13.92	7.59	30.38	18.99	3.80
	Overall Total	3796.00	844.00	22.23						

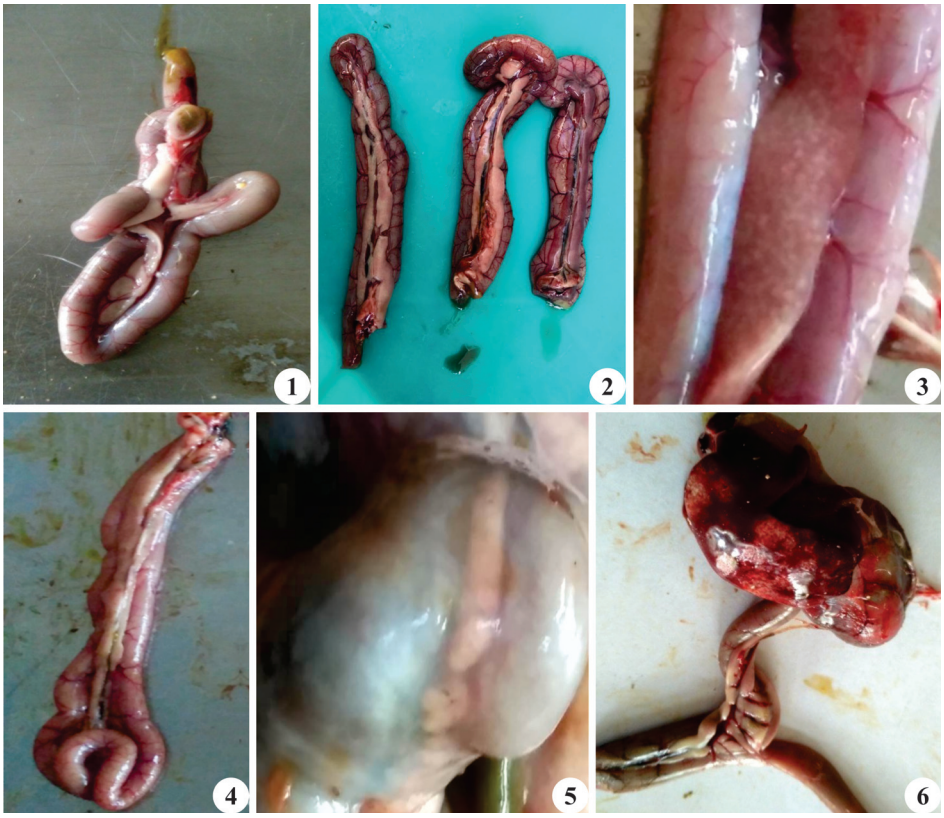

Fig. 1. Photograph showing bilaterally symmetrical mid segmental lateral deviation of duodeno-pancreatic complex along with bleached appearance of pancreas; **Fig. 2.** Photograph showing variable degree of folding and mottling of duodeno-pancreatic complex; **Fig. 3.** Photograph showing mottling with multifocal round to irregular whitish necrotic foci in pancreas; **Fig. 4.** Photograph showing terminal deformity of duodeno-pancreatic complex; **Fig. 5.** Photograph showing atrophy and bleached appearance of the pancreas in knotted intestine found in a case of cystadeno carcinoma; **Fig. 6.** Photograph showing mid segmental curving of duodeno-pancreatic complex with hyperplastic changes in pancreas.

detection of mycotoxin in the poultry feed during disease incidence. Ascarids were detected during post mortem examination of diseased birds, while coccidiosis was diagnosed on the basis of gross lesions in intestine and confirmed by detection of oocyst in the fecal examination by direct method. Non-infectious conditions like gout, chick oedema disease and nephrosis was confirmed on the basis of gross lesions.

The statistical analysis and test for significance were carried out by calculating Chi-square statistics for 2x2 contingency table. Whenever and wherever required, Yates correction was employed as per standard protocol described by Snedecor and Cochran[12]. All the data was entered in excel spread sheet and data was analyzed using SPSS software.

RESULTS

Gross pancreatic pathology

The major gross pathological findings of pancreas in decreasing order of frequency were observed as bleached appearance, congestion, mottled appearance with multifocal necrotic lesions, pancreatic deformity, atrophy, and hypertrophy. The incidence of pancreatic pathology within individual group of diseases is presented in decreasing order of frequency in Table 1. It is evident from the table that incidence of pancreatic involvement is significantly higher in all the listed viral diseases with highest incidence being observed in cases of IBD (94.74%) followed by IB (93.33%) and others. Amongst the bacterial diseases, pasteurellosis showed highest incidence of pancreatic pathology (90.48%). High incidence of pancreatic pathology was also observed in birds suffering from brooder's pneumonia (83.33%), chick oedema disease (88.46%) and suspected cases of mycotoxicosis (81.48%).

Bleached pancreas was characterized by marked white discoloration of the pancreas as against pinkish to pale yellowish color seen normally in pancreas (Fig. 1). Congested pancreas on the other hand developed dark pinkish to reddish pink discoloration (Fig. 2). Mottling of the pancreas was characterized by increased hardness in the consistency of pancreas with yellowish discoloration and formation of discrete multifocal white to creamy white, round necrotic lesions. These focal changes were either embedded in the parenchyma or showed discrete

nodular swelling (Fig. 3). Pancreatic deformity ranged from simple bending, curving, folding, coiling, wavy presentation, single twisting, and multiple twisting of duodeno-pancreatic complex. The primary deformity in all cases were seen in the pancreas which was mimicked by duodenum (Fig. 4). Atrophy of pancreas was characterized by reduction in the width of pancreas most of the time and in the length of pancreas in some of the cases (Fig. 5). Hyperplastic pancreas on the other hand was characterized by focal widening of the pancreatic mass leading to deflection in the associated duodenum (Fig. 6).

Bleached appearance of pancreas was prominent in diseases such as coccidiosis (82.76%), ascaridiasis, fowl pox (both 66.67%) and IBD (55.56%). Congestion of pancreas was most marked in chicken infectious anemia (71.43%), pneumonia (63.93%), chick edema disease (60.87%), coryza (54.17%) and IB (50%). Mottling was significantly high in cases of gout (66.67%), lymphoid leucosis (55%) and ascaridiasis (50%). Pancreatic deformity was found more in diseases like chick oedema disease (56.52%), colibacillosis (53.33%) and IBD (50%).

Microscopic pancreatic pathology

Prominent histopathological alterations of diseased pancreas in decreasing order of frequency were interstitial fibrosis, congestion, dissociation and individualization of acinar cells, multifocal necrosis, vacuolar degeneration with residual / apoptotic bodies, periductular fibrosis, and capsular thickening (Table 2). The degree and severity of fibrosis ranged from mild interstitial to more advanced fibrosis in the intercellular or peri-acinar region creating round island like lobular pattern. (Fig. 7). Staining of the pancreas suspected for fibrosis with MalloryTrichrome stain, prominently expressed distinct

Table 2. Incidence of histopathological lesions in the pancreas during different disease conditions of poultry.

Types of Diseases	Disease	Interstitial Fibrosis	Congestion	Individualization	Multifocal Necrosis	Vacuolar Degeneration	Periductular Fibrosis	Increased Zymogen Granules	Thickened Capsule
Viral	I.B.D.	42.86	42.86	28.57	28.57	14.29	28.57	0.00	0.00
	I.B.	14.29	42.86	42.86	71.43	42.86	28.57	0.00	0.00
	Cystadeno carcinoma	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
	Fowl Pox	100.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00
	Lymphoid leucosis	83.33	33.33	50.00	50.00	33.33	0.00	0.00	0.00
	Chicken infectious anaemia	60.00	50.00	30.00	60.00	30.00	10.00	0.00	30.00
	R.D.	49.02	33.33	45.10	21.57	9.80	5.88	25.49	5.88
Bacterial	Pasteurella	100.00	33.33	33.33	33.33	66.67	0.00	0.00	0.00
	Oophoritis	28.57	42.86	28.57	14.29	0.00	14.29	14.29	14.29
	CORYZA	66.67	66.67	66.67	33.33	100.00	0.00	0.00	0.00
	Enteritis	37.50	37.50	37.50	0.00	25.00	0.00	0.00	0.00
	Collibacillosis	40.00	40.00	20.00	40.00	60.00	20.00	0.00	40.00
	Yolk Sac Infection	55.00	35.00	25.00	15.00	15.00	10.00	10.00	0.00
	Pneumonia	33.33	66.67	66.67	66.67	33.33	0.00	0.00	0.00
Fungal	Brooder's Pneumonia	100.00	100.00	0.00	100.00	0.00	100.00	100.00	0.00
Parasitic	Ascaridiasis	50.00	50.00	0.00	0.00	50.00	0.00	0.00	0.00
Protozoal	Coccidiosis	40.00	70.00	10.00	40.00	50.00	30.00	20.00	0.00
Metabolic	Chick oedema disease	50.00	87.50	25.00	12.50	25.00	25.00	12.50	12.50
	Gout	100.00	50.00	0.00	0.00	0.00	0.00	0.00	0.00
Toxicological	Aflatoxicosis	40.00	60.00	20.00	40.00	0.00	0.00	20.00	0.00
	Ochratoxicosis	72.73	56.82	27.27	38.64	43.18	20.45	13.64	2.27
Total Number of Condition Specified (Out of 204 pancreas examined)		111.00	95.00	66.00	63.00	57.00	27.00	27.00	11.00
Overall %		54.41	46.57	32.35	30.88	27.94	13.24	13.24	5.39

pattern of fibrosis in the interstitial, interlobular and periductular space (Fig. 8). Congestion of exocrine pancreas was characterized by vascular lumen being completely or partially filled with excess of erythrocytes (Fig. 9). Individualization of acinar cells in exocrine pancreas was characterized by dissociation of cells and disruption of acinar pattern (Fig. 10). Multifocal Necrosis was characterizedby loss of acinar cells with infiltration of mononuclear cells or heterophils.Vacuolar degeneration was characterized by presence of small or large vacuoles in the acinar cells. In advanced cases 70-80% acinar cells showed vacuolations. Often round or oval residual bodies were found in the vacuoles. These residual bodies are suggestive of apoptotic cells (Fig. 11). The capsule of pancreas sometimes showed considerable thickening due to proliferation of connective tissue, infiltration of inflammatory cells, sub capsular necrosis and individualization of acinar cells.

Pancreatic pathology in different varieties of birds

Highest susceptibility to pancreatic pathology was shown by Kadaknath birds (85.71%) followed by 75.34% in broiler birds (Table 3). The disease wise pancreatic involvement in different varieties of birds is presented in Table 1. Jharsim, a variety developed by CVSc & AH, Ranchi showed significant disease susceptibility and pancreatic pathology. Diseases such as brooder pneumonia, aflatoxicosis, IBD, pasteurellosis, visceral gout, oophoritis, chick oedema disease and cystadenocarcinoma in decreasing order of frequency showed pancreatic pathology in Jharsim birds (Table 3). The variation in pancreatic pathology between various poultry varieties wasmostly found significant (Table 6).

Season wise variation in pancreatic pathology

The highest percentage of pancreatic pathology was observed in monsoon season (28.84%) followed by summer (22.57%) and least i.e. 12.92% in winter season (Table 2). Higher susceptibility during summer season to pancreatic pathology was registered in diseases such as IB, pasteurellosis, oophoritis, coryza, colisepticaemia, brooder pneumonia, coccidiosis, chick edema disease, gout, nephrosis and ascaridiasis. In winter season, the pancreatic pathology was high for diseases like IB, IBD, coryza, chick oedema disease and lymphoid leucosis. Monsoon month showed higher pancreatic pathology in diseases like IBD, IB, fowl pox, pasteurellosis, enteritis, brooder pneumonia, ascaridiasis, chick edema disease and suspected mycotoxicosis. Seasonal variation of disease incidence and pancreatic pathology was highly significant (Table 7).

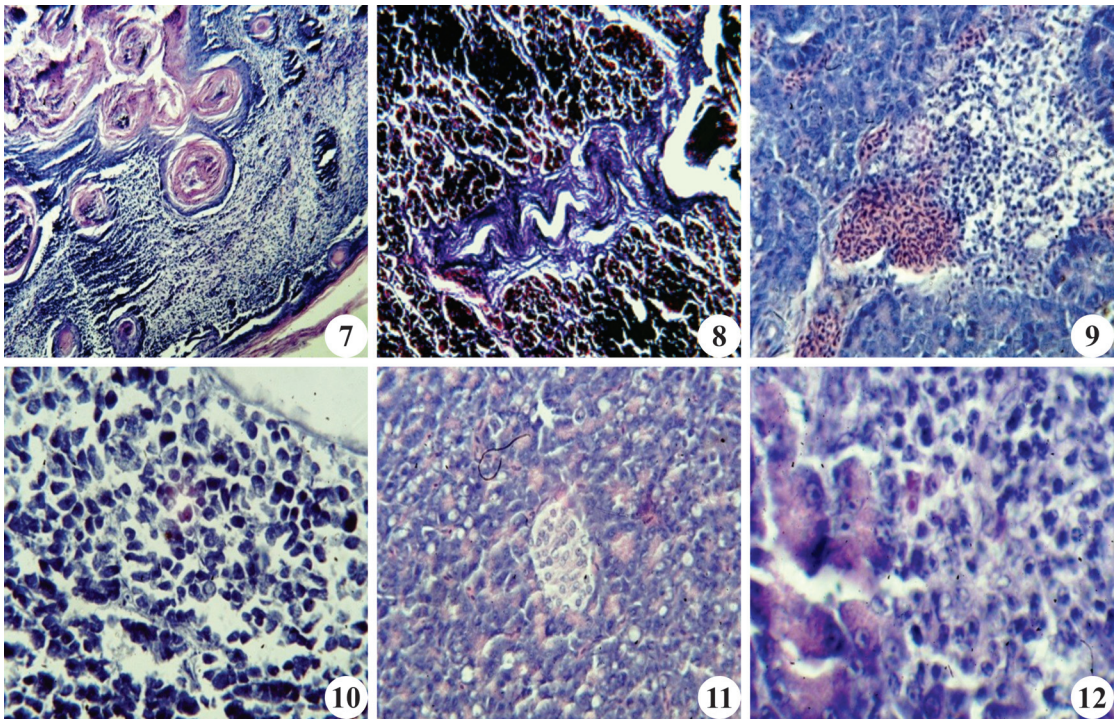

Fig. 7. Photomicrograph showing massive sub capsular necrosis of acinar cells and infiltration of inflammatory cells with island of completely necrosed acinar cells surrounded by thick band of connective tissue (H&E, 400X); **Fig. 8.** Photomicrograph showing thick band of proliferating fibrous connective tissue invading the interstitium in exocrine pancreas (Mallory Trichrome stain - 400X); **Fig. 9.** Photomicrograph showing congestion of blood vessels, necrosis and infiltration of inflammatory cells in exocrine pancreas (H&E, 400X); **Fig. 10.** Photomicrograph showing marked dissociation / individualization of acinar cells with mild interstitial fibrosis (H&E, 400X); **Fig. 11.** Photomicrograph showing widespread vacuolar degeneration of acinar cells with unaffected islet of Langerhans (H&E, 400X); **Fig. 12.** Photomicrograph showing residual bodies with clean halo around it in acinar cells (H&E, 400X).

Table 3. Incidence of pancreatic pathology in poultry varieties during different diseases.

S.No.	Disease	Kadaknath			Broiler			PB2			Cross Breeds			Jharsim			Desi			D. Red			Vanraja		
		No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%	No. of birds examined	No. Pancreas affected	%
1.	Yolk Sac Infection	0.00	0.00	0.00	10.00	9.00	90.00	15.00	1.00	6.67	5.00	3.00	60.00	190.00	50.00	26.32	55.00	14.00	25.45	7.00	2.00	28.57	47.00	8.00	17.02
2.	Collibacillosis	0.00	0.00	0.00	1.00	1.00	100.00	18.00	5.00	27.78	2.00	0.00	0.00	13.00	4.00	30.77	6.00	3.00	50.00	1.00	0.00	0.00	6.00	2.00	33.33
3.	Brooder Pneumonia	0.00	0.00	0.00	4.00	4.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	100.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4.	Chick Oedema Disease	0.00	0.00	0.00	0.00	0.00	0.00	7.00	7.00	100.00	0.00	0.00	0.00	9.00	7.00	77.78	2.00	2.00	100.00	8.00	7.00	87.50	0.00	0.00	0.00
5.	Pneumonia	0.00	0.00	0.00	0.00	0.00	0.00	21.00	6.00	28.57	22.00	7.00	38.89	231.00	30.00	12.99	58.00	13.00	22.41	5.00	0.00	0.00	31.00	5.00	16.13
6.	Coryza	1.00	1.00	100.00	5.00	5.00	100.00	11.00	3.00	27.27	0.00	0.00	0.00	29.00	10.00	34.48	7.00	5.00	71.43	2.00	0.00	0.00	0.00	0.00	0.00
7.	Enteritis	0.00	0.00	0.00	0.00	0.00	0.00	18.00	17.00	94.44	6.00	4.00	100.00	108.00	47.00	43.52	24.00	9.00	37.50	1.00	0.00	0.00	20.00	0.00	0.00
8.	Lymphoid Leucosis	0.00	0.00	0.00	3.00	3.00	100.00	5.00	2.00	40.00	0.00	0.00	0.00	18.00	9.00	50.00	5.00	5.00	100.00	1.00	1.00	100.00	0.00	0.00	0.00
9.	Gout	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.00	5.00	83.33	1.00	1.00	100.00	2.00	0.00	0.00	0.00	0.00	0.00
10.	Nephrosis	4.00	3.00	75.00	0.00	0.00	0.00	28.00	12.00	42.86	24.00	2.00	9.52	197.00	57.00	28.93	33.00	3.00	9.09	5.00	2.00	40.00	1.00	0.00	0.00
11.	Oophoritis	0.00	0.00	0.00	3.00	0.00	0.00	0.00	0.00	0.00	5.00	1.00	50.00	17.00	14.00	82.35	11.00	8.00	72.73	2.00	0.00	0.00	0.00	0.00	0.00
12.	I.B.	0.00	0.00	0.00	12.00	12.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	2.00	1.00	50.00	1.00	1.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
13.	R.D.	2.00	2.00	100.00	14.00	5.00	35.71	69.00	19.00	27.54	191.00	48.00	18.18	1107.00	139.00	12.56	499.00	59.00	11.82	242.00	17.00	7.02	0.00	0.00	0.00
14.	I.B.D.	0.00	0.00	0.00	3.00	3.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	16.00	15.00	93.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15.	Pasteurellosis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	18.00	16.00	88.89	3.00	3.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
16.	Ascaridiasis	0.00	0.00	0.00	0.00	0.00	0.00	4.00	1.00	25.00	2.00	1.00	0.00	13.00	4.00	30.77	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17.	Coccidiosis	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	0.00	0.00	0.00	0.00	106.00	9.00	8.49	0.00	0.00	0.00	27.00	20.00	74.07	0.00	0.00	0.00
18.	Fowl Pox	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	3.00	3.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
19.	Aflatoxicosis/ Mycotoxicosis	0.00	0.00	0.00	18.00	13.00	72.22	0.00	0.00	0.00	0.00	0.00	0.00	9.00	9.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20.	Cyst adeno Carcinoma	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	100.00	3.00	2.00	66.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
21.	Chicken Infectious Anaemia	0.00	0.00	0.00	0.00	0.00	0.00	33.00	20.00	60.61	0.00	0.00	0.00	9.00	1.00	11.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Overall	7.00	6.00	85.71	73.00	55.00	75.34	234.00	93.00	39.74	258.00	67.00	25.97	2103.00	430.00	20.45	711.00	129.00	18.14	303.00	49.00	16.17	105.00	15.00	14.29

Age wise variation in pancreatic pathology

Maximum pancreatic pathology was registered in grower birds (33.69%) followed by chicks (20.06%) and adult birds (19.54%). Grower birds showed increase susceptibility to develop pancreatic pathology in cases of IB, fowl pox, pasteurellosis, enteritis, colibacillosis, chick oedema disease and suspected mycotoxicosis. The diseases which produced greater percentage of pancreatic pathology in chicks were IBD, IB, brooder pneumonia, chick edema disease, gout and suspected mycotoxicosis. In adult birds, diseases like IB, cystadenocarcinoma, pasteurellosis, visceral gout and mycotoxicosis produced greater incidence of pancreatic abnormalities (Table 5). Chi square difference in pancreatic pathology between Chicks v/s Grower and Grower v/s Adult was highly significant (Table 7).

Effect of sex on pancreatic pathology

The incidence of pancreatic pathology in different disease conditions was higher in female (52.49%) as compared to male (47.51%). The diseases which showed higher pancreatic pathology in female birds includes IBD, cystadenocarcinoma, oophoritis, enteritis, yolk sac infection, pneumonia, brooder pneumonia, coccidiosis, gout and mycotoxicosis. In male birds, pancreatic involvement was higher in diseases such as chick oedema disease, lymphoid leucosis, IB, RD, pasteurellosis, fowl pox and chicken infectious anemia (Table 6).

DISCUSSION

The pancreas play vital role in metabolic activities of poultry. Its dysfunction under different disease conditions might be an important contributory factor in the manifestation and outcome of diseases of poultry, particularly with respect to body weight and general health. Only little work has been done to study the pancreatic pathology under different disease conditions in domestic and wild birds.

Present yearlong study on 3796, birds irrespective of age, sex and breed revealed distinct pancreatic pathology in 22.23% of total birds examined. Our finding is similar to the report of Qamar *et al.*[13] who

Table 4. Incidence of pancreatic pathology in different diseases during different season.

S.No.	Disease	Winter			Summer			Monsoon		
		No. of birds affected	Pancreas affected	%	No. of birds affected	Pancreas affected	%	No. of birds affected	Pancreas affected	%
1.	Yolk Sac Infection	143.00	12.00	8.39	136.00	61.00	44.85	50.00	14.00	28.00
2.	Collibacillosis	18.00	1.00	5.56	5.00	3.00	60.00	24.00	11.00	45.83
3.	Brooder Pneumonia	1.00	0.00	0.00	2.00	2.00	100.00	3.00	3.00	100.00
4.	Chick Oedema Disease	4.00	4.00	100.00	4.00	4.00	100.00	18.00	15.00	83.33
5.	Pneumonia	214.00	17.00	7.94	62.00	30.00	48.39	92.00	14.00	15.22
6.	Coryza	6.00	6.00	100.00	8.00	8.00	100.00	41.00	10.00	24.39
7.	Enteritis	87.00	11.00	12.64	47.00	25.00	53.19	43.00	41.00	95.35
8.	Lymphoid Leucosis	8.00	4.00	50.00	13.00	13.00	100.00	13.00	3.00	23.08
9.	Gout	3.00	0.00	0.00	6.00	6.00	100.00	0.00	0.00	0.00
10.	Nephrosis	66.00	7.00	10.61	14.00	14.00	100.00	212.00	58.00	27.36
11.	Oophoritis	8.00	2.00	25.00	23.00	17.00	73.91	7.00	4.00	57.14
12.	I.B.	3.00	3.00	100.00	4.00	4.00	100.00	8.00	7.00	87.50
13.	R.D.	0.00	0.00	0.00	1824.00	273.00	14.97	300.00	20.00	6.67
14.	I.B.D.	1.00	1.00	100.00	0.00	0.00	0.00	18.00	17.00	94.44
15.	Pasteurellosis	0.00	0.00	0.00	20.00	18.00	90.00	1.00	1.00	100.00
16.	Ascaridiasis	14.00	1.00	7.14	6.00	4.00	66.67	1.00	1.00	100.00
17.	Coccidiosis	136.00	23.00	16.91	2.00	2.00	100.00	0.00	0.00	0.00
18.	Fowl Pox	0.00	0.00	0.00	0.00	0.00	0.00	4.00	3.00	75.00
19.	Aflatoxicosis/ Mycotoxicosis	0.00	0.00	0.00	13.00	9.00	69.23	14.00	13.00	92.86
20.	Cyst adeno Carcinoma	0.00	0.00	0.00	2.00	2.00	100.00	2.00	1.00	50.00
21.	Chicken Infectious Anaemia	0.00	0.00	0.00	2.00	0.00	0.00	40.00	21.00	52.50
	Overall	712.00	92.00	12.92	2193.00	495.00	22.57	891.00	257.00	28.84

have described pancreatic histopathological alterations in 16.65% birds. It the present study, group wise consideration showed highest incidence of pancreatic pathology in fungal (83.33%) and metabolic (82.86%) diseases which mainly included disease conditions like brooder pneumonia, gout and chick oedema disease. However, consideration of individual disease revealed thatin viral diseases like IBD, IB and bacterial disease like pasteurellosis, the incidence of pancreatic involvement was even higher i.e. 94.74%, 93.33% and 90.48% respectively. Such high incidence of pancreatic pathology in many of the disease conditions highlights the importance of pancreatic abnormality as one of the major contributory factor in disease development and poor production performance of such birds.

A number of reports are there which has observed significant pancreatic involvement and pathological alteration in different viral diseases of birds such as avian adenovirus, paramyxovirus type I, herpesvirus, Newcastle disease virus, avian influenza virus, avian parvo virus, avian encephalomyelitis virus, paramyxo-3 virus, polyoma virus, turkey viral hepatitis, West Nile virus, and psittacid herpes virus[14-21]. No previous reports are available on some of the viral diseases examined in the present study such as IBD, IB, fowl pox, lymphoid leucosis and chicken infectious anemia.

In the present study, the major gross pathological changes of pancreas consisted of bleached appearance, congestion, mottling with multifocal pale yellowish often somewhat minutely nodular necrotic foci, pancreatic deformity, pancreatic atrophy and hypertrophy. Somewhat similar description of gross pancreatic pathology in birds has also been reported by Rantzer[22] and Majumdar[8]. Out of all the gross pathological changes mentioned, highest incidence was recorded for bleached and congested appearance (36.97%). Similar to our finding bleached appearance of diseased pancreas as a major gross pathological alteration has also been reported by Qamar[13].

Lower incidence of pancreatic deformity in young chicks is understandable because of poorly developed pancreatic and duodenal structures. In adult due to

Table 5. Incidence of pancreatic pathology in different age group during different diseases.

S.No.	Disease	Chick			Grower			Adult		
		No. of birds affected	Pancreas affected	%	No. of birds affected	Pancreas affected	%	No. of birds affected	Pancreas affected	%
1.	Yolk Sac Infection	329.00	87.00	26.44	0.00	0.00	0.00	0.00	0.00	0.00
2.	Collibacillosis	41.00	12.00	29.27	4.00	3.00	75.00	2.00	0.00	0.00
3.	Brooder Pneumonia	6.00	5.00	83.33	0.00	0.00	0.00	0.00	0.00	0.00
4.	Chick Oedema Disease	18.00	18.00	100.00	8.00	5.00	62.50	0.00	0.00	0.00
5.	Pneumonia	337.00	60.00	17.80	27.00	1.00	3.70	4.00	0.00	0.00
6.	Coryza	47.00	23.00	48.94	2.00	1.00	50.00	6.00	0.00	0.00
7.	Enteritis	136.00	53.00	38.97	23.00	17.00	73.91	18.00	7.00	38.89
8.	Lymphoid Leucosis	0.00	0.00	0.00	14.00	8.00	57.14	20.00	12.00	60.00
9.	Gout	5.00	4.00	80.00	2.00	0.00	0.00	2.00	2.00	100.00
10.	Nephrosis	107.00	50.00	46.73	127.00	25.00	19.69	58.00	4.00	6.90
11.	Oophoritis	0.00	0.00	0.00	0.00	0.00	0.00	38.00	23.00	60.53
12.	I.B.	5.00	4.00	80.00	9.00	9.00	100.00	1.00	1.00	100.00
13.	R.D.	943.00	49.00	5.20	295.00	100.00	33.90	886.00	140.00	15.80
14.	I.B.D.	19.00	18.00	94.74	0.00	0.00	0.00	0.00	0.00	0.00
15.	Pasteurellosis	0.00	0.00	0.00	7.00	5.00	71.43	14.00	14.00	100.00
16.	Ascaridiasis	11.00	3.00	27.27	7.00	2.00	28.57	3.00	1.00	33.33
17.	Coccidiosis	25.00	5.00	20.00	91.00	23.00	25.27	22.00	1.00	4.55
18.	Fowl Pox	1.00	0.00	0.00	3.00	3.00	100.00	0.00	0.00	0.00
19.	Aflatoxicosis/ Mycotoxicosis	10.00	8.00	80.00	14.00	11.00	78.57	3.00	3.00	100.00
20.	Cyst adeno Carcinoma	0.00	0.00	0.00	1.00	0.00	0.00	3.00	3.00	100.00
21.	Chicken Infectious Anaemia	29.00	16.00	55.17	13.00	5.00	38.46	0.00	0.00	0.00
	Overall	2069.00	415.00	20.06	647.00	218.00	33.69	1080.00	211.00	19.54

well-developed organ, increased resistance to disease process and stronger connective tissue framework, the probability of pancreatic deformity becomes less. In grower birds which have shown higher incidence of pancreatic pathology, the developmental changes in the organ are expected to be in active state. There is more proliferation of parenchymal cells and less support of connective tissue stroma. It is a well-established fact that avian exocrine pancreas has poor interacinar and intralobular connective tissue framework[23]. Thus, any persistent injury to pancreas may bring about tissue necrosis and fibrous connective tissue proliferation which is often quite extensive as evidenced in histopathological study and histochemical demonstration of massive interstitial fibrosis. The resisting nature of interstitial and interlobular pathological fibrosis and proliferating nature of developing and regenerating exocrine pancreatic tissue may lead to distortion in the shape of pancreas which brings about concomitant spontaneous twisting of duodenal loop, matching each and every curve being developed in diseased pancreas.

Pancreatic deformity has the potential to bring about obstructive changes in pancreatic duct and blood supply. Any obstruction in the ductular passage may interfere with secretary function of exocrine pancreas. Degenerative changes and proliferative changes have been observed in ductular epithelium of birds examined along with periductular fibrosis on histopathological examination. Congestion has also been a consistent change in pancreatic parenchyma in the present study. All these points are clearly indicative of abnormal pancreatic function, thereby directly or indirectly responsible for poor body weight gain due to improper digestive and absorptive processes. The magnitude of pancreatic deformity exhibited by birds suffering from different disease conditions had been the high point of present study. Except for report of parvo virus initiated terminal bending of pancreas and duodenum published by Nunez[14], no report is available on the incidence of pancreatic deformity both in domestic and wild birds. In the present study, highest incidence of pancreatic deformity was observed in chick edema disease and colisepticaemia. Both these conditions mainly affect

Table 6. Incidence of pancreatic pathology in different sex during different diseases.

S.No.	Disease	Pancreas Affected	Male	Percent	Female	Percent
1.	Yolk Sac Infection	87.00	33.00	37.93	54.00	62.07
2.	Collibacillosis	15.00	7.00	46.67	8.00	53.33
3.	Brooder Pneumonia	5.00	2.00	40.00	3.00	60.00
4.	Chick Oedema Disease	23.00	14.00	60.87	9.00	39.13
5.	Pneumonia	61.00	28.00	45.90	33.00	54.10
6.	Coryza	24.00	12.00	50.00	12.00	50.00
7.	Enteritis	77.00	30.00	38.96	47.00	61.04
8.	Lymphoid Leucosis	20.00	16.00	80.00	4.00	20.00
9.	Gout	6.00	2.00	33.33	4.00	66.67
10.	Nephrosis	79.00	39.00	49.37	40.00	50.63
11.	Oophoritis	23.00	0.00	0.00	23.00	100.00
12.	I.B.	14.00	8.00	57.14	6.00	42.86
13.	R.D.	289.00	157.00	54.33	132.00	45.67
14.	I.B.D.	18.00	5.00	27.78	13.00	72.22
15.	Pasteurellosis	19.00	10.00	52.63	9.00	47.37
16.	Ascaridiasis	6.00	3.00	50.00	3.00	50.00
17.	Coccidiosis	29.00	12.00	41.38	17.00	58.62
18.	Fowl Pox	3.00	2.00	66.67	1.00	33.33
19.	Aflatoxicosis/ Mycotoxicosis	22.00	8.00	36.36	14.00	63.64
20.	Cyst adeno Carcinoma	3.00	1.00	33.33	2.00	66.67
21.	Chicken Infectious Anaemia	21.00	12.00	57.14	9.00	42.86
	Overall	844.00	401.00	47.51	443.00	52.49

Table 7. Chi-square analysis for comparison of pancreatic pathology in different poultry varieties, season, age group and sex.

Comparison	X^2 statistic	Comparison	X^2 statistic
Jharsim v/s PB2	45.1374**	Desi v/s Kadaknath	16.5407**
Jharsim v/s desi	1.7712NS	Desi v/s Vanraja	0.9369NS
Jharsim v/s D. Red	3.036NS	Desi v/s Cross Breeds	7.1846**
Jharsim v/s Kadaknath	14.3661**	Desi v/s Broiler	120.5914**
Jharsim v/s Vanraja	2.3591NS	D. Red v/s Kadaknath	18.1579**
Jharsim v/s Cross Breeds	4.2165*	D. Red v/s Vanraja	0.2097NS
Jharsim v/s Broiler	122.7464**	D. Red v/s Cross Breeds	8.1551**
PB2 v/s Desi	45.7024**	D. Red v/s Broiler	102.9359**
PB2 v/s D. Red	37.7172**	Kadaknath v/s Vanraja	17.5394**
PB2 v/s Kadaknath	4.187*	Kadaknath v/s Cross Breeds	9.3788**
PB2 v/s Vanraja	21.6378**	Kadaknath v/s Broiler	0.0228NS
PB2 v/s Cross Breeds	10.6096**	Vanraja v/s Cross Breeds	5.8254**
PB2 v/s Broiler	28.2417**	Vanraja v/s Broiler	67.2784**
Desi v/s D. Red	0.5708NS	Cross Breeds v/s Broiler	59.6008**
Winter v/s Summer	31.0463**	Chick v/s Grower	51.2712**
Winter v/s Monsoon	58.9113**	Chick v/s Adult	0.1209NS
Summer v/s Monsoon	13.5183**	Grower v/s Adult	43.4337**
Male v/s Female	4.1801*		

**Highly Significant, p<0.01; *Significant, p<0.05; NS - Non-significant, p≥0.05

grower birds and are associated with increased intra-abdominal pressure due to accumulation of transudate or exudates which may have interfering effect on thesecretary functions of pancreas.

Bleached appearance of pancreas was most prominently seen in coccidiosis, ascaridiasis, fowl pox, IBD and nephrosis. Anemia may constitute important underlying factor in giving bleached appearance to pancreas. Other changes such as thickening of capsule, increased interstitial fibrosis, atrophy, significant degenerative and infiltrative changes may play important role in development of bleached appearance. Congestion of pancreas is related to passive hyperemia associated with disorders such as pneumonia, viraemia and septicaemic conditions in cases of infectious bronchitis, pasteurellosis, colisepticaemia, yolk sac infection and oophoritis. Congestion was also prominently seen in chick edema disease which is characterized by increased intra-abdominal pressure and interference with venous return. Congestive changes have the potential to bring about significant histopathological alteration due to hypoxic injury such as cellular swelling and necrosis of parenchymal cells. These changes were very much evident in the histopathological study of pancreas which exhibited congestion. Similar to our findings, congestive changes in cases of pancreatitis has also been reported[24].

Atrophic changes in pancreatic parenchyma were not a consistent finding and constituted a minor pathological change characterized by shrunken white pancreas giving lesser gross visibility of the organ during postmortem examination. It was most evident in cases of ovarian cystadenocarcinoma of adult hen. Hyperplasia of pancreas was seen in a very small percentage (2.61%) of birds. Exact cause of hyperplastic changes could not be ascertained. It may be considered as developmental anomaly or increased local availability of growth

factors.

The most frequent histopathological alteration found in the study was interstitial fibrosis. It was most evident in the cases of pancreatic deformity. In advanced lesions, pronounced interlobular and periductular fibrosis was observed. Pancreatic stellate cells have also been recognized as an important participant in mammalian pancreatic fibrogenesis[25,26]. In case of pancreatic injury, the stellate cells undergo myofibroblastic transformation with capability to synthesize fibrillar collagen. However, this mechanism in avian pancreatic fibrosis needs further exploration and confirmation. Interstitial fibrosis may play important role in development of pancreatic deformity since fibrous tissue being firm and strong may resist expansive growth of pancreatic parenchyma particularly in grower birds with resultant curving, bending or twisting of duodeno-pancreatic complex.

Congestion was a consistent change in most of the disease; if persistent it can bring about hypoxic damage to the parenchymal cells. Hypoxia often leads to cellular swelling, vacuolar degeneration and necrosis. These changes were found in the acinar cells of pancreas showing hyperemia. Increased intra abdominal pressure in conditions like chick edema disease, oophoritis, egg peritonitis, colisepticaemia or mushy chick disease brings about passive hyperemia due to compression of inferior vena cava.

Significant number of diseased pancreas exhibited individualization of acinar cells. Any deficiency or toxicity which can specifically damage the basement membrane of acini and adjacent interstitium will lead to acinar cell dissociation. Residual bodies develop within the acinar cells[6]. The cells finally loose their polarity and get dissociated. Deficiency of essential nutrients like copper and selenium or zinc toxicosis has been reported to be the underlying factor in the development of individualization of acinar cells. It is quite possible that birds suffering from various infectious and metabolic diseases might have developed nutritional deficiencies, thus initiating loss of polarity or individualization of acinar cells. The reason for individualization of acinar cells in poultry needs further exploration.

Histopathological study of diseased pancreas in the present investigation brought forward two more important microscopic alteration of relatively high frequency *viz.* multifocal necrotic lesion with infiltration of inflammatory cells and vacuolar degeneration of the pancreatic acinar cells with formation of residual bodies considered to be a manifestation of apoptotic changes characterized by shrinkage in size of the cells with or without pyknotic nuclei[27]. Residual bodies thus represent degenerated acinar cells often surrounded by clear halo. Similar description of pancreatic necrosis and associated lesions was also described[28]. Multifocal exocrine pancreatic necrosis was registered in all groups of diseases; however its incidence was significantly higher in viral diseases such as IB, fowl pox, lymphoid leucosis, Chicken infectious anaemia, RD and IBD. Affinity of viral diseases for pancreatic parenchyma with multifocal necrosis of exocrine pancreas has also been reported in mammalian diseases such as canine distemper, canine parvo virus infection, felid herpes virus infection, FMD, classical swine fever, paramyxo virus infection, and type I avian influenza[28,29].

Thickened capsule in the present study has been observed as associated change with inflammatory conditions in pancreas. Thickened capsule may give pancreas a bleached blood. Thickening of capsule and associated fibrous changes may make the organ susceptible to adhesive changes with organ of digestive system as seen in lymphoid leucosis or cystadenocarcinoma where the pancreas becomes completely embedded in thickened capsule from where connective tissue infiltrated the parenchyma to bring about degenerative and necrotic changes in pancreatic acini.

Thus it is evident from the study that both gross and histopathological changes of diseased pancreaswere mostly non-specific in nature and doesn't carry any significant diagnostic value for a particular disease condition. However, their presence is clear indication of pancreatic dysfunction and its effect on the health of the birds.

Kadaknath and broiler birds showed greatest susceptibility to pancreatic abnormalities. The stressful conditions of overcrowding, poor ventilation and poor litter management in which broiler birds are mostly kept, might lead to stress related immunosupression and higher incidence of different disease conditions and pancreatic abnormalities. Other supposedly disease resistant indigenous breeds like jharsim also showed significant susceptibility to pancreatic abnormalities, though to a lesser extent as compared to broiler birds. High incidence of pancreatic pathology (46.65%) in broiler birds has also been reported by Qamer[13] and Nunez[14]. Amongst bacterial diseases, *E.coli* infection showed consistent pancreatic lesion in different poultry varieties. Metabolic diseases like chick oedema disease and gout showed discrete pancreatic lesions in all varieties except in broiler birds. Most of the reports pertaining to avian pancreatic pathology are in wild birds or game birds such as pigeon, ostrich, macaw, cockatiel, free flying trumpeter, swan, wild water fowl etc[5,18,30].

Incidence of pancreatic pathology was higher in monsoon than summer and least in winter. In monsoon high humidity, poor ventilation, high moisture, and poor control of flies and insects could make the birds

stressful and prone to suffer from disease conditions. Managemental lapses due to heat stress during summer season must be the underlying condition for compromised immune status and higher incidence of disease. In winter, the weather showing low humidity and lack of heat stress, keep birds healthy and immunologically strong to resist invasion by disease-causing agents.

Age of the birds has definite effect on pancreas, younger birds have poorly developed pancreas whereas old birds show significant replacement of exocrine and endocrine pancreas by fibrous connective tissue as a senile change. Under both the age groups, pancreatic function is sub optimal[8.21]. In the present investigation, highest incidence of pancreatic pathology was observed in grower birds (33.69%) while the incidence of pancreatic pathology was in the range of 19-20% in both chicks and adult birds. Higher incidence of pancreatic pathology in chicks were observed in cases of chick oedema disease, IBD, and brooder pneumonia; whereas in grower, it was observed in cases of IB, fowl pox and colibacillosis while in adult birds, incidence was higher in cases of pasteurellosis, mycotoxicosis, cystadenocarcinoma, lymphoid leucosis and oophoritis. It is important to consider the fact that nature of disease varies amongst the three age groups due to age susceptibility and other important contributory factors such as management, biosecurity, vaccination practices etc. No clear-cut observation on age susceptibility to various diseases and related pancreatic pathology has been mentioned in any of the previous reports, though disease incidences and pancreatic pathology in chicks and grower has been reported[21,19].

The variation in the incidence of pancreatic pathology in different sex was found to be significantly higher in female. Study revealed that diseases in which pancreatic pathology was found to be higher in female birds were IBD, colibacillosis, yolk sac infection, pneumonia, and brooder pneumonia. For rest of the diseases with pancreatic pathology, the incidence was higher in male or equal in both the sexes such as coryza and ascaridiasis. No clear-cut predisposing factors could be ascertained for variation in disease incidence and pancreatic pathology under the influence of sex.

Thus, we can conclude that frequent pathology of pancreas in different disease conditions of poultry bird suggests suboptimal functioning of pancreas. Therefore, it would be beneficial to add pancreatic enzyme as a feed supplement in poultry as well as vitamin A and vitamin E to support the regenerative process in damaged pancreas of diseased birds as a routine practice for early recovery of the flock from disease condition and production loss.

ACKNOWLEDGEMENTS

The study was supported by grant made available by Birsa Agricultural University, Kanke. The authors are thankful to Director Research, BAU, Kanke for providing necessary support and to the Dean, CVSc & AH, BAU, Ranchi for providing necessary facilities to carry out the study.

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Indian J. Vet. Pathol., 48(1) : 62-66, 2024: DOI: 10.5958/0973-970X.2024.00009.9

SHORT COMMUNICATION

Prevalence and antibiotic susceptibility of bacteria isolated from poultry of different farms of Rajasthan, India

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Received: 8.8.2023; Accepted: 19.9.2023

ABSTRACT

The study was conducted to determine the antibiotic susceptibility patterns of the bacteria producing diseases in poultry. A thorough post mortem examination of poultry carcasses obtained from different regions of Southern Rajasthan was done and samples were collected from liver, heart blood and intestine. Isolation and Identification of Bacterial pathogen was done as per the standard procedure and they were further examined for *In-vitro* antibiotic susceptibility by the disc diffusion method. The bacteria that were isolated from poultry were *Escherichia coli*, *Salmonella species*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumonia.* Prevalence of *E. coli* infection was highest. Majority of the bacterial isolates were sensitive to Cefixime, Amikacin, Ceftriaxone and Cefotaxime. So, these drugs may be used as the drug of choice against these bacterial isolates. Resistance was observed against Ampicillin, Co-Trimoxazole, Tetracycline, as well as towards Gentamicin, Amoxyclav and chloramphenicol. It is concluded that prevalence of *E. coli* infection was highest in poultry and multi-drug resistance bacteria are increasing rapidly. This suggests implementing better management practices and prudent use of antibiotics in poultry to minimize the antibiotic resistance in animals and its transmission to humans.

Keywords: Antibiotic resistance, antibiotic susceptibility, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella sps.*, *Staphylococcus aureus*

How to cite this article : Damor, P.K., Kumari, M., Sharma, D.K., Yadav, V. and Limbat, R. 2024. Prevalence and antibiotic susceptibility of bacteria isolated from poultry of different farms of Rajasthan, India. Indian J. Vet. Pathol., 48(1) : 62-66.

The poultry industry is emerging as the world's largest market. The total poultry in the country is 851.81 million in 2019, increased by 16.8% over previous censusas reported by Basic Animal Husbandry Statistics. During the last few decades, there is an emphasis on intensive rearing of poultry. Despite ofsignificant advances, diseases are still a major constraint for profitable poultry production. Infectious and non-infectious diseases take a heavy toll from all types of poultry enterprises. Amongst the various disorders, enteric diseases accounts for high mortality in poultry and the common gastrointestinal tract conditions reported are colibacillosis, salmonellosis, coccidiosis, Ranikhet disease, necrotic enteritis, and fatty liver syndrome[1]. Besides, indiscriminate use of antibiotics in the poultry sector is leading to antibiotic drug resistance which is a global concern. There are reports on clear cut and rapid transmission of antimicrobial resistance from food animals to humans[2]. Therefore, this study was designed to observe the prevalence of bacterial diseases in poultry farms, and to assess their antibiotic susceptibility pattern.

Post mortem examination of 84 poultry carcasses obtained from different regions of Southern Rajasthan was conducted in the Department of Veterinary Pathology. Samples were aseptically collected from the liver, intestine, and heart blood. The samples were then processed for isolation and identification of bacteria by the standard procedure of[3].The samples (heart blood, liver and intestine) collected during postmortem examination were incubated in Nutrient broth at 37^{O}C for 24 to 48 h. The growth obtained was then streaked on blood agar (BA) and Mac Conkey's Lactose agar (MLA) and incubated at 37^{O}C for 24 h. The bacterial growth so obtained was studied for colony characteristics and hemolysis on various agar media as per the standard procedure described by[4].

For primary identification of bacteria, catalase test and oxidase test were performed. Besides, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization tests were also performed according to the standard procedure described by[4]. Gram's staining of a single pure colony was done for microscopic examination. After isolation, and complete biochemical characterization, the *Salmonella* and *E. coli* isolates were sent for serotyping to the National *Salmonella* and *Escherichia* center, (NSEC), Central Research Institute, Kasauli (HP), India.

Bacterial isolates obtained in the study were tested for *In-vitro* drug susceptibility by the disc

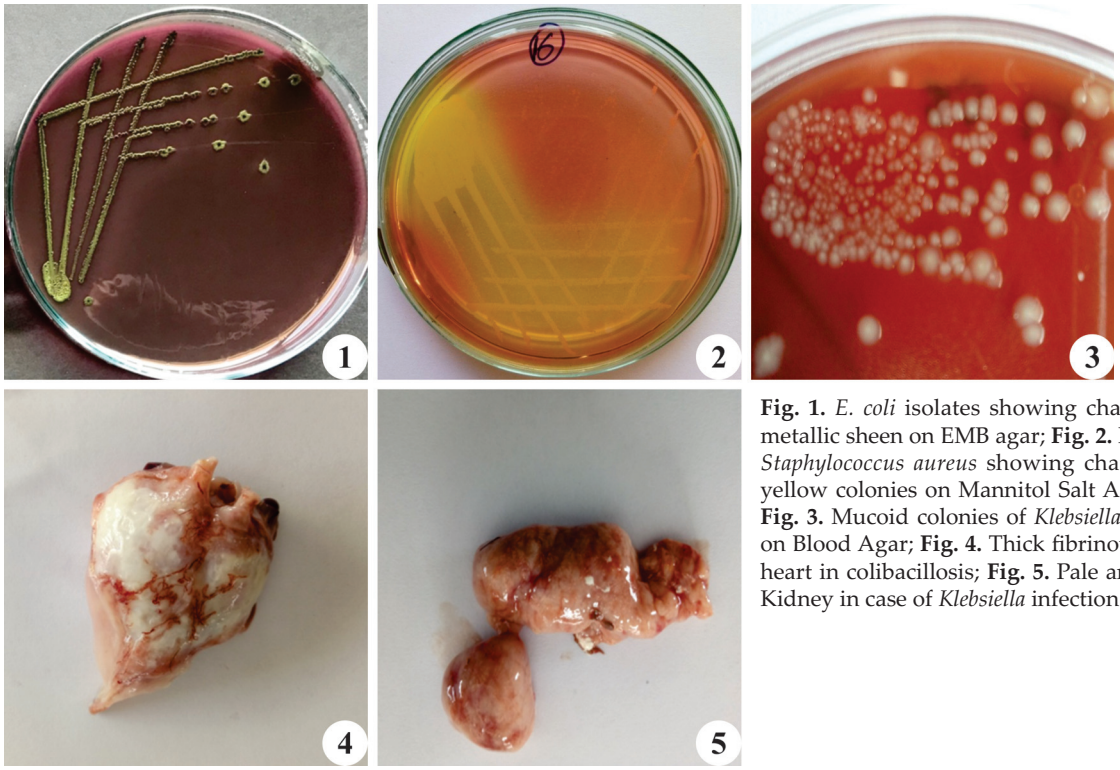

Fig. 1. *E. coli* isolates showing characteristics metallic sheen on EMB agar; **Fig. 2.** Isolation of *Staphylococcus aureus* showing characteristics yellow colonies on Mannitol Salt Agar (MSA); **Fig. 3.** Mucoid colonies of *Klebsiella pneumonia* on Blood Agar; **Fig. 4.** Thick fibrinous layer on heart in colibacillosis; **Fig. 5.** Pale and necrotic Kidney in case of *Klebsiella* infection.

diffusion method as suggested by[5]. A small amount of test culture was taken in a sterile platinum loop and transferred into a tube of Nutrient broth and then kept in an incubator at 35^{O}C for 2 to 5 hours. The broth culture was then evenly spread over the surface of Mueller Hinton agar plates. The antibiotic discs of standard concentrations (Ampicillin 10 mcg/disc, Cefixime 5 mcg/disc, Co-trimoxazole 25 mcg/disc, Gentamicin 10 mcg/disc, Amoxyclave 30 mcg/disc, Streptomycin 10 mcg/disc, Tetracycline 30 mcg/disc, Chloramphenicol 30 mcg/disc, Ciprofloxacin 5 mcg/disc, Cefotaxime 30 mcg/disc, Trimethoprim 5 mcg/disc, Ceftriaxone 10 mcg/disc) were then placed on the agar with a minimum 24 mm distance between two discs. The discs were gently pressed to have uniform close contact with the medium. These plates were then kept at 37^{O}C for 24 h. The results were recorded as sensitive (S) and resistant (R) on the basis of the zone size interpretation chart, provided by the manufacturer.

The bacteria isolated from the liver, intestine and heart blood of birds during the study period were *Escherichia coli* (70.31%), *Salmonella spp*. (12.5%), *Staphylococcus aureus* (9.37%), *Pseudomonas aeruginosa* (6.25%) and *Klebsiella pneumoniae* (1.56%) (Figs. 1, 2 and 3). The necropsy examination revealed that *Escherichia coli* infected poultry showed presence of fibrin layer on all the visceral organs particularly on heart and liver (Fig. 4). Purulent exudate was observed in the lumen of proventriculus & gizzard. In Salmonellosis, the characteristic lesions in poultry were hepatomegaly and multiple necrotic foci on firm and dark red liver. Spleen showed presence of small and grayish white necrotic foci. In heart, haemorrhages were evident and white nodular growths were seen on myocardium. Gross lesions observed in *Pseudomonas aeruginosa* infection were congestion in trachea, lungs, heart and intestines. Few necrotic foci on liver and mild fibrinous perihepatitis and pericarditis, and catarrhal enteritis ina few cases were seen. Infection of *Klebsiella pneumonia* revealed hepatomegaly along with multiple pale hard necrotic areas in the liver and heart, necrotic area in spleen and kidney, severe congestion & haemorrhages were noted in the large intestine & cloaca. In *Staphylococcus aureus* infection, haemorrhages and congestion were noted throughout the gastrointestinal tract and liver.

The results of serotyping revealed that predominant serotype of *E. coli* isolated was O83 (45.83%) followed by O120 (12.5%), O149 (8.33%), O11 (4.16%), O134 (4.16%), O7 (4.16%), O157 (4.16%), O9 (4.16%), rough (4.16%) and untypable (8.33%). *E. coli* serotypes O157 and O149 were found associated as a mixed infection with *Salmonella* spp. *E. coli* serotypes O83 and O149 were found associated with *P.aeruginosa* infection. *E. coli* serotypes O83 and O120 were found associated with *S.aureus*. *E. coli* infection was also found along with parasitic infection (roundworm and tapeworm).

Salmonella spp. were isolated from a total of 12.5% of cases. The result of the serotyping of Salmonella samples revealed three different serotypes viz. *S.* Typhimurium, *S.* Welteverden, *S.* Linderburg. However, *Staphylococcus*

aureus was isolated from 9.37% of cases. *P. aeruginosa* was isolated from 6.25% of cases. *K. pneumoniae* was reported from 1.56% of cases and identified based on culture characteristics and biochemical tests. Besides, *K. pneumoniae* was found along with infection of *S.* Typhimurium and parasitic infection (roundworm and tapeworm).

Antibiotic susceptibility tests revealed a varying degree of sensitivity to the chemotherapeutic agents (Table 1). The resistance of *E. coli* is seen against ampicillin, Co-Trimoxazole, tetracycline and cefotaxime although ciprofloxacin is still showing 50% sensitivity. The antibiotic susceptibility results of *Salmonella* spp. revealed sensitivity to most of the antibiotics tested except to Co-Trimoxazole and 50% to both Tetracyclin and Cefotaxime.

In most of the reports, *E. coli* is the most predominant bacteria isolated from poultry[6-9]. O83, O149, and O120 were also predominant serotypes isolated from northern India indicating a prevalence of the similar types of serotypes in northern and western India[10,11]. However, in addition to these, other serotypes (O1, O22, O37, O114, O118, O78, O75, O2, O6, and O111) were also isolated from north India[11,12]. It is observed that *E. coli* serotype O9 is prevalent in Southern Rajasthan as well as in Odisha and West Bengal indicating its countrywide presence[13,14]. The major concern is the presence of *E. coli* O157 isolate as causes severe disease in humans. The vast variation in other *E. coli* serotypes may be due to variation in geographical area, climate and host and managemental procedures practiced in these areas.

It has been reported that 17.78% of the flocks tested from seven states of India were positive for *Salmonella* sps.[15]. These were identified as *S*. Gallinarum and *S.* Pullorum. Highest seroprevalence of salmonellosis is reported from Karnataka state (21.73%), while the lowest from Haryana (8.86%)[16]. However, a lower incidence of salmonellosis (4.90% and 6.1%) was reported in West Bengal, India[17,18]. This indicated that salmonellosis was more prevalent in the southern states of India as compared to the other parts of the country. Serotypes of Salmonella identified in the present study were *Salmonella* Typhimurium, *S*. Linderburg, and *S.*Weltevreden. *S*. Typhimurium has been reported by other workers from different parts of India and is one of the commonest serotypes isolated in India[17,19,20]. *S. enterica* serovar Weltevreden is associated with the outbreak of foodborne gastroenteritis in humans reported from Mangalore, Kolkata, Pune[21-23] and even from Reunion island, France[24]. There is increasing occurrence of Non-Typhoidal Salmonella infections in humans due to *S*. Typhimurium and *S.* Weltevreden[25]and so there is urgent need to control these infections in poultry to reduce foodborne infections in humans. This suggests that strict biosecurity measures must be taken to reduce the occurrence of foodborne pathogens. *S*. Weltevreden is capable of persisting for long periods in soil and thus it is important to treat contaminated poultry manure[26].

The prevalence of *S. aureus* in Haryana (North India) is reported to be 5.63%[27] whereas 82% in coastal Andra Pradesh (South India)[28]. This indicates that the prevalence of *S. aureus* infection is lower in northern and western India as compared to Southern India. The prevalence of *P. aeruginosa* and *K. pneumoniae* noted was 6.25% and 1.56%, respectively. Similarly, other workers also reported a low incidence of these bacteria in poultry farms located in North India[10,27].

The resistance of E. coli to antibiotics is also observed in other parts of the world like California[28], Italy[29] and Bangladesh[30], and India[31] suggesting that it's a worldwide

Table 1. *In-vitro* antibiotic sensitivity of various bacteria (% sensitivity) isolated from different visceral organs and heart blood.

Drugs	***E. coli***	***Staphylococcus aureus***	***Salmonella* spp.**	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***
Ampicillin	27.58	14.28	80	00	100
Cefixime	84.48	71.42	90	100	100
Ceftriaxone	60.34	71.42	80	100	100
Co-Trimoxazole	22.41	00	00	00	50
Gentamicin	24.13	42.85	70	100	50
Amoxyclav	46.55	57.14	85	87.5	50
Streptomycin	84.48	57.14	75	50	100
Tetracycline	25.86	28.53	50	00	100
Chloramphenicol	32.75	28.57	85	50	50
Ciprofloxacin	50	00	90	50	00
Cefotaxime	25.86	85.71	50	100	100
Amikacin	91.37	85.71	75	100	100

public health issue. However,sensitivity to ceftriaxone was previously reported higher as compared to the present study indicating that *E. coli* may become resistant to this drug soon[10]. Low levels of resistance to gentamicin (12%) are observed in Iran[32] while in Tanzania, highest resistance is reported against Co-trimoxazole (65.8%) whereas Gentamicin was found sensitive (69.3%)[33]. Gentamicin is also reported to be highly sensitive in the years 2014 and 2015, whereas, in the present study, Gentamicin was found resistant to 75.87% of *E. coli* isolates showing the emergence of *E. coli* resistant to Gentamicin[34]. Moreover, antibiotic resistance reported in areas of Maharashtra, India is more as compared to the present study[35]. As per literature there is an increased incidence of *E. coli* resistant to Oxytetracycline and Fluoroquinolones in South East Asian Countries[36]. The increasing incidence of antibiotic resistance may include inappropriate and/or excessive use of antimicrobials. This suggests the need for judicious use of antibiotics in poultry.

The antibiotic susceptibility results of *Salmonella spp.* showed contrast to the results reported by other workers who observed sensitivity to cefotaxime and resistance to ciprofloxacin in Haryana state which may be due to the differences in the use of antibiotics in different regions and environmental conditions[37]. Similarly, high sensitivity to chloramphenicol and resistance to tetracycline[20] whereas it is observed that Non-Typhoidal *Salmonella* isolates in Vietnam were resistant (20-40%) against tetracycline, chloramphenicol, sulfamethoxazole-trimethoprim, and ampicillin[38]. In contrast to the results of the present study, some workers reported the resistance of *Salmonella* isolated from backyard poultry in West Bengal towards chloramphenicol, ciprofloxacin, gentamicin, and oxytetracycline[18]. This suggests the need for good managemental practices at farms and restriction of indiscriminate use of antibiotics in poultry. This variation in susceptibility of the bacteria to the same antibiotic in different areas of the country as well as the world suggests that there is a need to use area-specific and sensitive antibiotics.

The sensitivity patterns of *K. pneumonia* and *P. aeruginosa* indicates that with time the resistance of these bacteria is increasing towards antibiotics because those antibiotic agents which were earlier showing sensitivity are now 100% resistant[8,27].

None of the antibiotics were 100% sensitive to *S. aureus*. More or less similar sensitivity patterns have been reported by other workers[39-41]. The rapid increase in antibiotic resistance might be due to the misuse of antibiotics in poultry farms. The other risk factors could be over-prescribing of antibiotics, unnecessary use of antibiotics for growth and poor hygiene and sanitation practices.

It is therefore concluded that the majority of bacterial isolates were sensitive to Cefixime, Amikacin, Ceftriaxone and Cefotaxime. So, these drugs may be used as the drug of choice against these bacterial isolates. There is a need to implement better management practices and adhere to strict biosecurity measures along with the prudent use of antibiotics to minimize disease occurrence and consequent economic losses.

ACKNOWLEDGEMENTS

The authors acknowledge the support of Rajasthan University of Veterinary and Animals Sciences for providing infrastructure, equipment's and financial support for materials required for conducting the research work of first author as part of master's dissertation.

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Indian J. Vet. Pathol., 48(1) : 67-70, 2024: DOI: 10.5958/0973-970X.2024.00010.5

SHORT COMMUNICATION

Chronic hyperplastic ingluvitis: Synergistic effect of invasive Candida and Trichomonas in a desi chicken

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Received: 4.9.2023; Accepted: 16.9.2023

ABSTRACT

A desi chicken flock (3 months old) had 2% mortality every week with a history of stunted growth. Birds were supplemented with antibiotics from the first week of hatch. Postmortem examination was carried out on a carcass. Carcass was pale and thin. Crop mucosa was covered with thin, whitish papery layer with scattered erosions. The mucosa also revealed an irregular corrugated firm growth protruding into the lumen. Cytology revealed narrow budding yeasts and numerous flagellated protozoa. Histologically, crop revealed epithelial hyperplasia with papillary projections. Hyperplastic epithelial layer showed ballooning degeneration of cells and inflammatory infiltrations. Histochemistry showed *Candida albicans* with PAS stain and *Trichomonas gallinae* with Giemsa stain. *C. albicans* exhibited yeast and hyphal forms which occupied superficial epithelial cells whereas septate hyphae were found penetrating perpendicular to the mucosa. Giemsa stained tissue section showed varying morphology of *Trichomonas gallinae* in the lamina propria. This study reporting the chronic and combined infection of dimorphic fungi - *Candida albicans* and protozoal flagellate *Trichomonas gallinae* in a desi chicken due to continuous usage of antibiotics.

Keywords: *Candidiasis*, chicken ingluvitis, invasive mycosis, *Trichomoniasis*

How to cite this article : Prasath, N.B., Selvaraj, J. and Velusamy, R. 2024. Chronic hyperplastic ingluvitis: Synergistic effect of invasive Candida and Trichomonas in a desi chicken. Indian J. Vet. Pathol., 48(1) : 67-70.

Candida albicans and *Trichomonas gallinae* are the opportunistic pathogen of avian species occurring world-wide[1,2]. Both are commensals of upper digestive tract (UDT) of birds. Avian candidiasis caused by *Candida albicans* is a sporadic disease which causes UDT mycosis. Trichomoniasis caused by *Trichomonas gallinae* is a protozoan disease which causes canker of UDT in birds[3]. These commensals cause clinical diseases under stress and or when the immune system get compromized[4]. Apart, concurrent debilitating diseases, disturbance in normal digestive flora due to long term antimicrobial therapy, hypovitaminaosis A, aflatoxicosis and high stocking densities and poor management predispose to the above diseases[1,2]. Growth and penetration of yeast or hyphae of *Candida albicans* and binary fission of *Trichomonas gallinae* begin to produce pseudomembranous layer covering mucosa of esophagus and crop[3]. Chronic infection of both these infectious agents result in mucosal epithelial hyperplasia of upper digestive tract, poor feed intake and stunted growth[5]. Mortalities occur due to invasive and multisystemic derangements. The present case report documents a case of chronic ingluvitis due to combined pathology of *Candida albicans* and *Trichomonas gallinae* in a desi chicken under free range system of rearing.

A stock holding 500 desi chicken (3 months old) had 2% mortality per week since one month of age. Flock had a history of stunted growth and continuous supplementation of tetracycline and enrofloxacin from first week of hatch. A carcass was brought to Department of Veterinary Pathology, Veterinary College and Research Institute, Orathanadu, Thanjavur for necropsy. Systematic postmortem examination was carried out.

The carcass was thin and emaciated (Fig. 1) with prominent keel bone. Skeletal muscles were pale and thin. Bone marrow was pale. On internal examination, crop was empty and mucosa revealed a whitish, thin pseudomembrane layer covering over it which peeled off easily. A few scattered areas showed an irregularly circular erosions. Mucosa of the dependent part displayed a focal irregular firm raised corrugated area resembling sessile cauliflower-head (Fig. 2). Tissue impression and scrapings from crop were collected for cytological examinations. Tissue samples were collected in 10% formalin for histopathology. Cytological samples were examined as wet mount with lactophenal cotton blue. Tissue samples were processed as per standard paraffin embedding technique and 3µ sections were prepared and stained with haematoxylin and eosin. Duplicate sections were stained with Periodic acid Schiff and Giemsa stain to demonstrate *Candida albicans* and *Trichomonas*

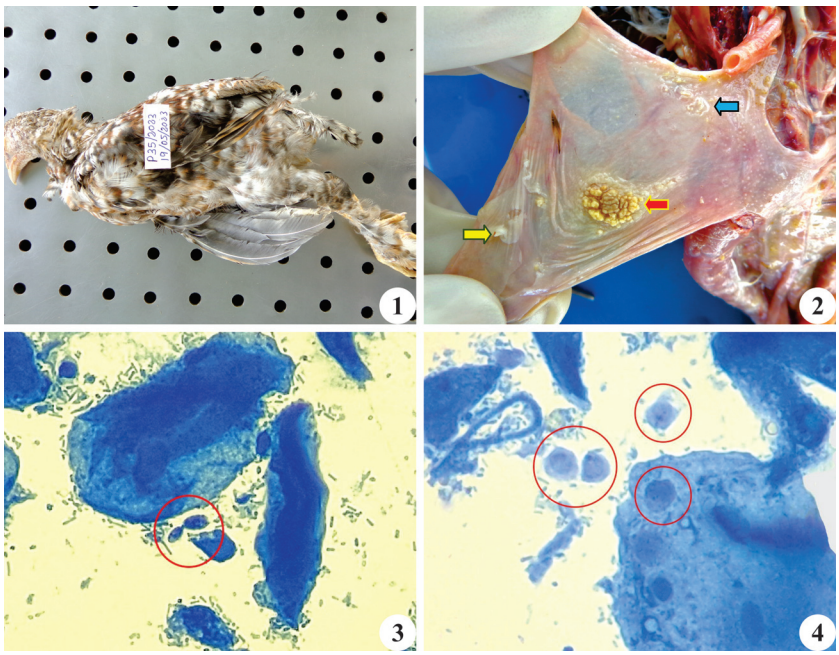


Fig. 1. Thin and emaciated carcass - Desi chicken; **Fig. 2.** Whitish pseudomembranous layer (yellow arrow), irregular erosions (blue arrow), an irregular, firm corrugated growth (red arrow) on the crop mucosa; **Fig. 3.** Budding yeast form of *Candida albicans* - Crop cytology - Lactophenol cotton blue stain x1000; **Fig. 4.** *Trichomonas gallinae* - Crop cytology - Lactophenol cotton blue stain x1000.

gallinae respectively as per standard technique[6].

Scrapings from the mucosa of crop showed numerous narrowly-based budding yeasts of *Candida albicans* (Fig. 3) and flagellate *Trichomonas gallinae* (Fig. 4). Histopathologically, crop revealed stratified squamous epithelial hyperplasia which were thrown into papillary projections into the lumen (Fig. 5). Epithelial cells were swollen with ballooning degeneration. Hyperplastic projections were infiltrated with mixed inflammatory cells at the center causing distention of epithelial folds (Fig. 6). Base of the hyperplastic papillae showed necrosis with scattered bacterial clumps (Fig. 7). The lamina propria showed many trophozoites of *Trichomonas gallinae*. Histochemistry with Giemsa stained tissue

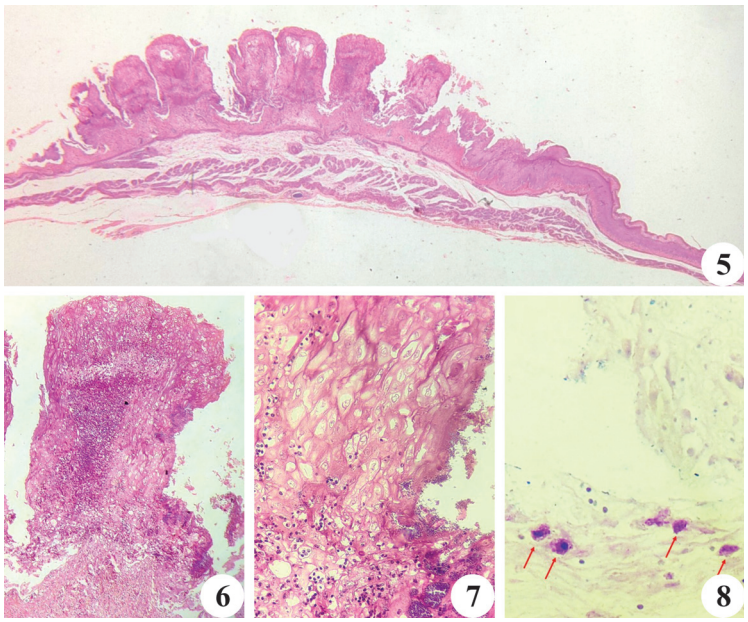
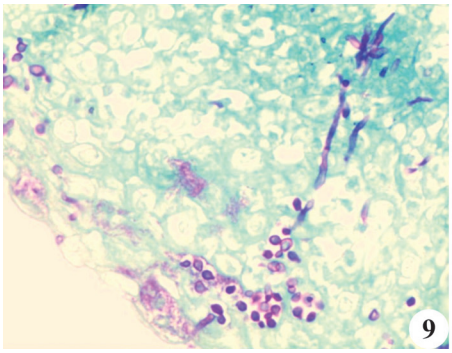


Fig. 5. Mucosal hyperplasia resembling "papillary projections" - Chronic ingluvitis - desi chicken (H&E x20); **Fig. 6.** Mucosal hyperplasia with severe inflammatory cell infiltration - crop - desi chicken (H&E x100); **Fig. 7.** Mucosal hyperplasia with ballooning of squamous epithelial cells and bacterial clumps - crop - desi chicken (H&E x400); **Fig. 8.** Trophozoites of *Trichomonas gallinae* within the hyperplastic epithelium - crop - desi chicken (Giemsa stain x400).



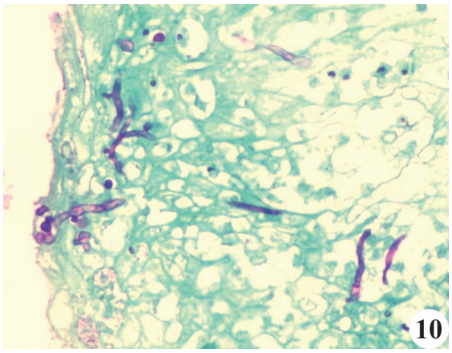


Fig. 9. Budding yeast bodies and penetrating hyphae of *Candida albicans* - crop mycosis - desi chicken - PAS with light green counter stain x400; **Fig. 10.** Septate hyphae penetrating deep into the mucosa - *Candida albicans* - crop mycosis - desi chicken - PAS with light green counter stain x400.

sections revealed many trophozoites of *Trichomonas gallinae* (Fig. 8) in the lamina propria and throng of inflammatory cell infiltrations. The Periodic acid Schiff stained tissue sections revealed many budding yeasts of *Candida albicans* over the surface of the hyperplastic epithelium and its septate hyphae found penetrated deep into the mucosa (Figs. 9 and 10).

The crop (ingluvies) is a sublingual thin walled muscular pouch/bag of esophagus in birds to store feed before digestion. Commensals of upper digestive tract affect the crop mucosa when innate immunity fails to comply and leads to ingluvitis. The most frequent causes are *Candidia albicans* and *Trichomonas gallinae*[1,2].

Candidiasis is a sporadic disease caused by an opportunistic pathogen *Candidia* spp especially *Candida albicans*. *Candida albicans* are polymorphic pathogen exhibiting budding yeast, hyphae and pseudohyphae structural forms depending on its growth environment[7]. Candidia takes any of the above three morphological forms in tissue. *Candida albicans* causes mycosis of UDT in poultry. Among, crop mycosis poses a significant threat to chicken rearing by way of growth disturbance, remarkable morbidity and mortality. More precisely, thickening of crop mucosa with yellowish-white, pseudomembranous deposits infer it as 'sour crop' or 'thrush'[8]. Invasion of *candida albicans* into deeper mucosal epithelium leads to hyperplasia[2]. Candidial hyphae penetrate the stratified squamous epithelium perpendicular to the mucosal surface of the present case was parallel with the earlier documented reports[9,10]. Mycelial invasion and subsequent epithelial hyperplasia causes necrosis and bacterial growth of the present observation was in concurrence with the previous documented evidence[5,11,12].

Alike candidiasis, trichomoniasis is a protozoan disease of birds caused by microaerophilic, flagellated parasite *Trichomonas gallinae* in the class Zoomastigophorea and order Trichomonadida. Avian trichomoniasis was reported in a variety of avian species and most commonly in columbiformes and falconiformes[13,14] and rarely in chicken. Pigeons act as primary host for worldwide transmission[4] and its disease is termed as *canker* whilst *frounce* in raptors[1]. It occupies surface epithelium of UDT and respiratory tract as commensal. The protozoa divides by binary fission and can invade tissues causing necrosis, when the innate immune mechanism is lost. *Trichomonas gallinae* exhibited its morphology as flagellates and trophozoite[3].

The morphological features observed in this study was in parallel with previous reports[1,3,15]. The flagellated *Trichomonas* were observed as free motile form and occupied the lumen of UDT. They were characterized by the presence of a single karyomastigont, five to six flagella and an undulating membrane[3,15]. The trophozoite form observed in the crop tissue showed morphological variation as ovoidal to pyriform with size ranged from 7-11µm. The nucleus was ovoid with a size measuring 2.5-3 µm[1]. The above morphological descriptions of many free flagellated protozoa were observed in cytology and trophozoites with varying morphology were recognized deeper in hyperplastic epithelium. The morphology of trophozites were identical with previous documented reports[16]. Albeit, Trichomonas occupy as free moving flagellate as commensal in UDT above gizzard, it invades deep into tissue to cause malady when disease evading mechanism get adjusted and or compromised. Further, it was documented that *Trichomonas* suggested to release glycosidase, neuraminidase and certain peptidases. These proteolytic enzymes were responsible for tissues damage and might be responsible for the present crop pathology[17,18].

Though umpteen predisposing factors line up, chronic usage of antibiotics is designated as a major factor for the present case. It disturbs the gut flora and contributes to tissue damages by commensal of gastrointestinal tract in chicken. It was suggested that the antibiotics may cause competent exclusion of bacteria and favors exuberant growth of candida and trichomonos[1] which was resumed

to be true in the present study. Extended and repeated usage of antibiotics in the present study paved a way for colonization, invasion, epithelial hyperplasia and necrosis by *Candida albicans* and *Trichomonas gallinae*. Histochemical observation with obvious tissue seething of *Candida albicans* and *Trichomonas gallinae* refuted the pathological changes were primarily owed by these pathogens as a consequence of immune compromise. In the end, crop pathology of present case, engendered reduced feed consumption, improper feed storage and digestion. Ultimately, the affected bird suffered from inanition which paves way for death.

This report documented the chronic ingluvitis due to combined infection of *Candida albicans* and *Trichomonas gallinae* in desi chicken. This report also alerts the poultry farmers to forgo autonomy in usage of antibiotics which hamper healthy poultry rearing and production. Authors suggested to circumspect and get professional guidance before employing antibiotics for poultry rearing. Elseway, we conclude it as "being good or bad, commensals become pathogen of interest".

ACKNOWLEDGEMENT

I extend my gratitude to Dr S. Sathesh Kumar, The Professor and Head, Department of Veterinary Gynaecology and Obstetrics, VCRI, Orathanadu for granting permission to use photomicrographic facility. We profoundly thank Tamil Nadu Veterinary and Animal Sciences University for the facilities provided.

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Indian J. Vet. Pathol., 48(1) : 71-73, 2024: DOI: 10.5958/0973-970X.2024.00011.7

SHORT COMMUNICATION

Outbreak of duck viral enteritis in the Cauvery delta region of Tamil Nadu

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Received: 7.9.2023; Accepted: 6.10.2023

ABSTRACT

Necropsy was conducted on three ducks, which were reared in the Cauvery delta region in a flock containing 3000 birds and were not vaccinated. Tissue samples like intestine, oesophagus, spleen, kidney and liver were collected for polymerase chain reaction (PCR) and histopathology. Grossly, oral cavity and oesophageal mucosa showed adhesion of diphtheritic membrane. Liver revealed multiple white necrotic foci. Epicardium showed petechial haemorrhages. Intestine mucosa revealed annular band haemorrhage, multiple petechiael haemorrhages, annular band with yellowish diphtheritic adhesion and multiple diphtheritic button ulcers of 2-3 mm diameter. PCR showed amplification produces band size at 446 bp using specific primer showed positive for duck viral enteritis virus. Histopathologically, intestinal mucosa revealed fibrinous exudate with desquamated epithelial cells, clumps of bacteria and inflammatory cells. Based on the gross lesions, PCR and histopathological lesions, the present case was confirmed as duck viral enteritis.

Keywords: Duck, Duck viral enteritis, pathology, PCR

Duck plague is an acute, highly contagious and fatal disease of domestic ducks and water fowls[1]. The first report of duck plague was recorded in Netherlands in 1923 and later from other countries[2]. Duck plague is caused by Anatid herpes virus 1 belonging to *Herpesviridae* family, *alpha herpesvirinae* sub family and genus *Mardi virus*[3]. In India, the disease outbreak was recorded in West Bengal[4], Karnataka[5], Uttar Pradesh[6], Kerala[7], Tamil Nadu[8-9] and Assam[10]. More than 25 outbreaks were recorded in various districts of Assam state in India during the period from August 2012 to December 2015[11]. The present study reports the occurrence of duck viral enteritis outbreak in the Cauvery delta region of Tamil Nadu.

Three ducks were received for necropsy at the Department of Veterinary Pathology, Veterinary College and Research Institute, Orathanadu. These ducks were reared in the Cauvery delta region with flock containing 3000 birds which were not vaccinated. During necropsy, gross lesions were recorded. Tissue samples like intestine, oesophagus, spleen, kidney and liver were collected in sterile container for polymerase chain reaction (PCR). The tissue samples like oesophagus, intestine, lungs, proventriculus, liver, pancreas, kidney, spleen and bursa of Fabricious were collected and fixed in 10% formalin for histopathological examination. The tissues samples were routinely processed, sectioned (4 µm thickness) and stained with Haematoxylin and Eosin (H&E) stain[12].

Grossly, oral and oesophageal mucosa showed adhesion of yellowish diphtheritic membrane. (Fig. 1). Liver revealed multiple white necrotic foci on entire surface. (Fig. 2). Heart showed petechial haemorrhages on the epicardium (Fig. 3). Lungs, trachea, ovary and kidney were congested. In one bird intestine mucosa revealed annular band haemorrhages (Fig. 4). Intestinal mucosa in another bird revealed annular band haemorrhage, multiple petechiael haemorrhages, annular band with yellowish diphtheritic adhesion and also multiple diphtheritic button ulcers of 2-3 mm diameter (Fig. 5).

How to cite this article : Thilagavathi, K., Selvaraj, J., Jaisree, S., Ramya, R., Prasath, N.B. and Prabu, P.C. 2024. Outbreak of duck viral enteritis in the Cauvery delta region of Tamil Nadu. Indian J. Vet. Pathol., 48(1) : 71-73.

The DNA was extracted from the homogenized tissue samples (intestine, oesophagus, spleen, kidney and liver) by Phenol chloroform method of DNA extraction to rule out duck viral enteritis. PCR reaction was carried out as per reference[13] from the extracted DNA to rule out the Duck viral enteritis virus (DVEV). Primers for DVEV DNA-directed DNA polymerase gene (Forward primer - 5′-GAA-GGC-GGG-TAT-GTA-ATG-TA- 3′and reverse primer - 5′-CAA-GGC-TCT-ATT-CGG-TAA-TG 3′) was used in this study The total reaction mixture contained - 12.5 µl amplicon Red Dye master mix, 10 pM forward

Fig. 1. Oral cavity and oeophageal mucosa showing adhesion of yellowish diphtheritic membrane; **Fig. 2.** Liver revealing multiple white necrotic foci on entire surface; **Fig. 3.** Epicardium shows petechial haemorrhages.

and reverse primers each, 2 µl of template DNA and nuclease free water up to 25 µl. PCR was performed in a Bio-Rad thermo cycler with the following conditions: 94^{O}C for 2 min followed by 35 cycles each of denaturation at 94^{O}C for 1 min, annealing at 55^{O}C for 1 min, synthesis at 72^{O}C for 2 min and final extension at 72^{O}C for 7 min. The PCR product was electrophoresed on 1.5% agarose gel in Tris EDTA buffer stained with 0.5µg/ml of ethidium bromide and visualized under Bio-Rad gel doc system, for desired size of band with DNA ladder (100bp DNA Ladder, BIO-HELIX). The result was positive expected amplicon size of 446 bp was seen for the specific primers (Fig. 6).

Histopathologically, intestinal mucosa revealed fibrinous exudate with desquamated epithelial cells and inflammatory cells. (Fig. 7). In another bird, intestinal mucosa showed adhesion of fibrinous exudate with clumps of bacteria and inflammatory cells (Fig. 8). Oesophageal mucosa showed ulceration with focal necrosis of gland. Liver showed diffuse moderate sinusoidal congestion. Pancreas revealed focal necrosis. Lungs, kidneys and ovary showed moderate congestion. Spleen showed multifocal congestion with mild lymphoid depletion. The bursa of Fabricious revealed moderate lymphoid depletion (Fig. 9).

Lesions of DVE are associated with disseminated intravascular coagulopathy and necrotic/degenerative changes in mucosa and submucosa of gastrointestinal tract in lymphoid and parenchymatous organs. These collective lesions, when present, are diagnostic of DVE[14]. Grossly, diphtheritic lesion in oesophagus was in agreement with earlier reports[9,10,14,15]. Necrotic foci in the liver were in agreement with earlier findings[14-15]. Epicardial haemorrhages were in agreement with previous reports[10,14,15]. Intestinal annular bands appear as intensely reddened rings visible from external and internal surfaces. Later, the entire band becomes dark brown and tends to separate at its margins from the mucosal surface. The multifocal necrosis of gut-associated lymphoid tissue causes ulceration covered by fibrinous pseudomembranes[14]. Similar lesions of intestinal annular of band haemorrhages, diphtheritic lesions and button ulcers were observed in the present report. The confirmatory diagnosis by PCR was in accordance with earlier reports[9,13,15]. Histopathological lesions like diphtheritic enteritis were similar to earlier reports[14]. Lymphoid depletion in the bursa of Fabricious was in agreement with recent experimental report[15]. Congestion and haemorrhage in parenchymatous organs were similar to earlier reports[9,14,15].

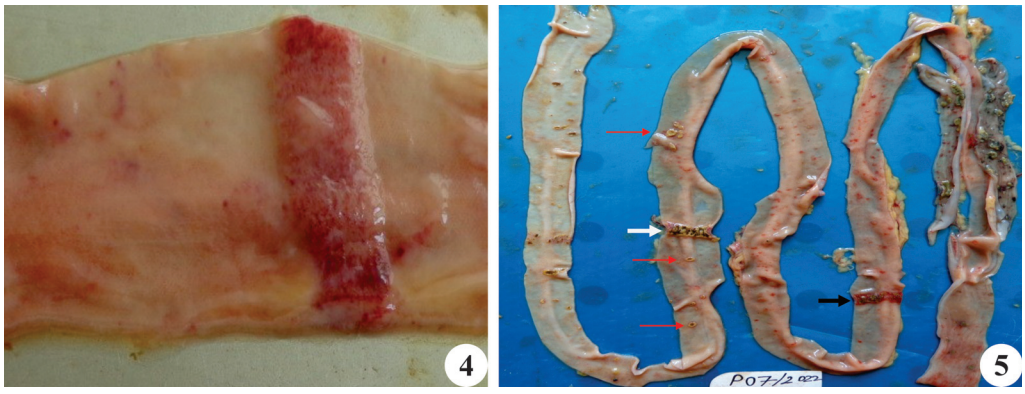

Fig. 4. Intestinal mucosa showing annular band haemorrhages; **Fig. 5.** Intestinal mucosa showing annular band haemorrhage (black arrow), multiple petechial haemorrhages, annular band with yellowish diphtheritic adhesion (white arrow) and button ulcers (red arrows).

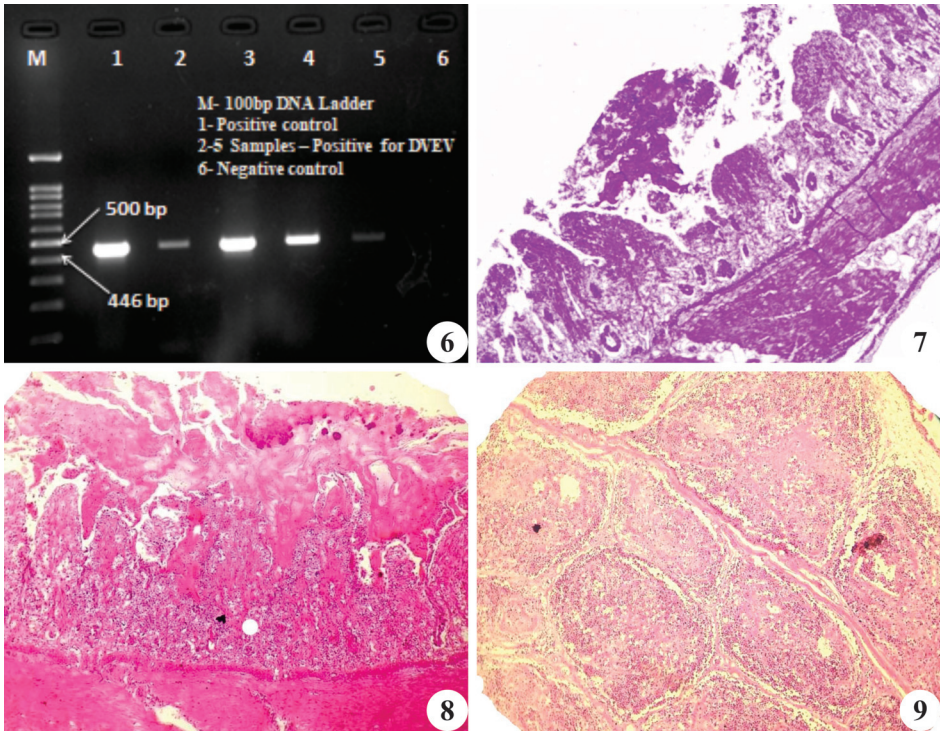

Fig. 6. PCR product showing amplification at 446 bp positive for duck viral enteritis virus; **Fig. 7.** Intestinal mucosa shows fibrinous exudate along with desquamated epithelial cells and inflammatory cells (H&E x40); **Fig. 8.** Intestinal mucosa shows adhesion of fibrinous exudate with clumps of bacteria and inflammatory cells (H&E x40); **Fig. 9.** Bursa showing moderate lymphoid depletion (H&E x100).

The present study reports the occurrence of duck viral enteritis infection in Cauvery delta region of Tamil Nadu. The affected ducks suffered from diphtheritic lesions in oral cavity, oesophagus and intestine resulting in decreased feed intake and body weight which led to increased mortality with severe economic loss to the farmers who were rearing ducks. Hence, the duck rearing farmers and veterinarians need to be aware of vaccination for duck viral enteritis in ducks and other waterfowls.

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Indian J. Vet. Pathol., 48(1) : 74-77, 2024: DOI: 10.5958/0973-970X.2024.00012.9

CASE REPORT

Pathology of unilateral squamous cell carcinoma of horn in a Himachali Pahari cow - A case study

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Received: 9.8.2023; Accepted: 11.9.2023

ABSTRACT

Squamous cell carcinoma (SCC) of horn is relatively more common and a highly malignant neoplasm, known to affect cattle. The present communication describes a case of well differentiated unilateral SCC of the horn in a Himachali Pahari cow. A ten year old cow was presented to Veterinary Polyclinic Lalhri, Dist. Una Himachal Pradesh with a history of bleeding growth around the base of right horn. Grossly, the growth was spongy, pinkish, cauliflower like in appearance with areas of surface ulceration and hemorrhages. Giemsa stained touch impression cytological smears revealed large number of malignant squamous epithelial cells occurring either individually or in clusters exhibiting hyperchromasia, marked pleomorphism, anisocytosis and anisokaryosis suggestive of SCC. Tissue sample from the ulcerated growth was collected in 10% neutral buffered formalin for histopathological examination. Histopathological analysis revealed cords and nests of proliferating neoplastic cells consisting of immature polyhedral cells at the periphery and eosinophilic laminated keratin pearls at the centre separated by thin fibrous tissue stroma confirmed it for well differentiated form of SCC. Based on cytological and histopathological findings besides special staining the case was confirmed as well differentiated squamous cell carcinoma of horn in a Himachali Pahari cow.

Keywords: Himachali Pahari cow, horn cancer, keratin pearl, squamous cell carcinoma

How to cite this article : Thakur, M., R. and Patil, R.D. 2024. Pathology of unilateral squamous cell carcinoma of horn in a Himachali Pahari cow - A case study. Indian J. Vet. Pathol., 48(1) : 74-77.

Squamous cell carcinoma (SCC) is the second most common form of skin cancer. The incidence of SCC has been reported to be 80% in cattle and 3% in buffalo[1]. SCC is a malignant tumor of epidermal cells. These tumors grow slowly, but are aggressive in nature. However, they do not metastasize to the regional lymph nodes. Among all tumors of cattle, SCC of horn is relatively more common and a highly malignant neoplasm, known to affect cattle[2]. Horn cancer is a common condition in cattle in India affecting approximately one percent of population. Horn cancer is generally unilateral and more common in cattle aged around 5-10 years with more prevalence in long horned cattle[3].

The predisposing factors for occurrence of horn cancer are considered to be multifactorial. Irritation due to yoke, trauma, tying the rope at the base of the horn, rubbing against hard object, fighting, genetic predisposition, paints, solar radiation, viruses, either alone or in combination, have been reported as the etiological factors. Sunlight is probably the most important carcinogenic stimulant for these tumors. High incidence of horn cancer in castrated bullocks indicates possible role of reproductive hormonal imbalance in the induction of tumor[4].

The most consistent clinical signs are frequent head shaking, tilting to the affected area, bending of affected horn and increase nasal discharge on the affected side in advance cases[5]. These tumors are painful and fill the horn core and may infiltrate to the frontal sinuses. Bullocks are more susceptible than cows[6]. Tumor appears as a cauliflower like growth with surface ulceration and bleeding[5].

Diagnosis of cancer, which is a prime requirement to take up treatment, is achieved in the field of oncology mainly by morphological and microscopic examinations including cytology, histopathology and immunohistochemical techniques. SCC originating from squamous epithelial cells presents varying features from incomplete carcinoma in intraepidermal form to highly malignant tumor type in its invasive form, exhibiting different degrees of differentiation in member cells of its progeny[7]. Grossly, SCC occurs as a nodular or ulcerative lesion presenting as red firm plaque to a cauliflower like ulcerated mass[8] and may be seen in any organ of the body lined by epithelium like skin, eye, horn, oral, nasal cavities, tongue, esophagus, lung, penis, vagina and footpad[9].

The present communication describes cytological and histopathological findings of horn cancer in a Himachali Pahari cow. A ten year old Himachali Pahari cow was presented to Veterinary

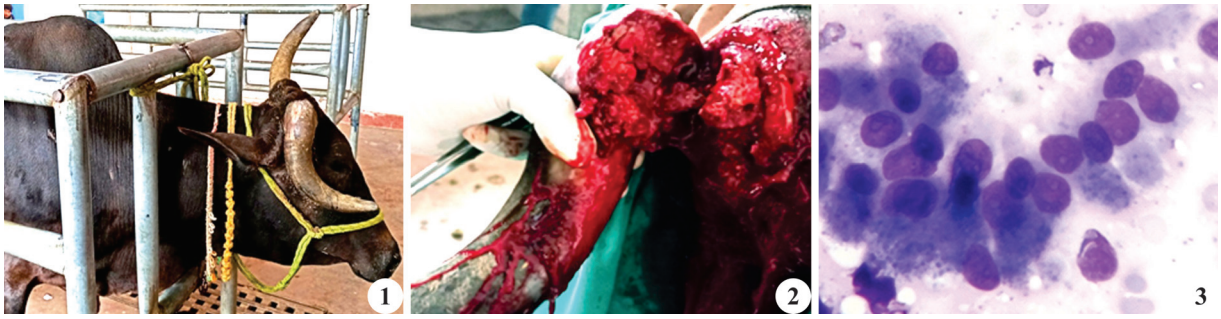

Fig. 1. Swelling and bending of right horn from base; **Fig. 2.** Spongy, friable, pinkish, irregular, cauliflower shaped bleeding growth at base of broken right horn with superficial necrosis and ulcerations; **Fig. 3.** Neoplastic squamous cells in clusters showing hyperchromasia, cellular pleomorphism and prominent and multiple nucleoli (Giemsa stain x1000).

Polyclinic Lalhri, Dist. Una, Himachal Pradesh, with a history of foul smelling mass attached to the base of the broken right horn with frequent bleeding and head shaking (Fig. 1). On gross examination, the mass was spongy, friable, pinkish, cauliflower like in appearance with superficial necrosis and surface ulcerations which bled easily (Fig. 2). Touch impression cytology smears were prepared from the mass and stained with Giemsa. For histopathological examination tissue sample was collected in 10% neutral buffered formalin. After fixation, tissue samples were processed and stained by routine Hematoxylin and eosin (H&E) as per standard protocol[10]. Slides were viewed at different magnifications using light microscope.

Grossly, the mass appeared to be spongy, friable, pinkish, irregular, cauliflower shaped with superficial necrosis and surface ulcerations. These observations were in conformity with the earlier findings[5]. A complete blood cell count analysis revealed normal hemoglobin, hematocrit and total erythrocyte count. However, total leukocyte count (TLC) was slightly elevated with marked neutrophilia. Cytological smears revealed large number of malignant squamous cells occurring either individually or in clusters. The cells were pleomorphic, round to caudate in shape exhibiting hyperchromasia, prominent anisokaryosis and anisocytosis. Anisokaryosis characterized by nuclei, varying from pyknotic to large type, variable nuclear to cytoplasmic ratio, prominent and multiple nucleoli, binucleation, frequent mitotic figures and perinuclear vacuolation were observed (Fig. 3). Such observations were in accordance with the previous observations[7,8]. Further, asynchronous cytoplasmic and nuclear maturation, varying number of keratinized squamous cells depending upon the degree of differentiation of SCC was also observed. The cytoplasm of keratinized cells appeared bluish to purplish in Giemsa stained smears. These results were well supported with the previous findings[11]. Cytological smears also revealed a large number of polymorphonuclear and mononuclear cells (Fig. 4). This observation was similar to earlier findings[12] and was obvious as tumor mass was ulcerative or inflamed with suppuration in the present study.

On histopathological examination the well differentiated invasive type SCC encountered in the present study was featured by cords or nests of proliferating neoplastic cells consisting of immature polyhedral cells at the periphery and eosinophilic highly lamellated keratin pearls at the centre (Figs. 5 and 6). The amount of keratin was abundant in well differentiated SCC. The proliferating cells revealed hyperchromasia, severe cellular pleomorphism, large vesicular nuclei, prominent and multiple nucleoli, increased mitotic activity and prominent intercellular bridges (Figs. 7 and 8).

Similar observations have been reported earlier[13]. The perinuclear halo or vacuolation observed in the present study was attributed to the presence of colorless keratohyaline granules as reported earlier[2]. The lamellated keratin in Masson's trichrome appeared brick red (Fig. 9). This special stain also facilitated in demarcating the amount of connective tissue that varied from moderate to marked. The connective tissue was particularly abundant around invading cords in deeper areas of the neoplasm (Fig. 9). These findings are in accordance with the previous workers[5,6] who observed fibroplasia or desmoplasia to be abundant around penetrating epithelial cells in the deeper areas of the neoplasm. Further the focal to multifocal areas of necrosis and inflammatory infiltration with lymphocytes, plasma cells and neutrophils was in concordance with previous observations[13].

Based on gross, cytological and histopathological examinations, the mass was confirmed as well differentiated squamous cell carcinoma in a Himachali Pahari cow. In bovine, skin and soft tissue tumors are more common. A study conducted by[13] revealed that among cattle tumors, squamous cell carcinoma comprised around 17.54%. 6000 horn cancer cases, 93% cases were observed in working bullock as reported[14]. The incidence, predisposing factors of horn cancer in bovine and recorded that higher incidence may be

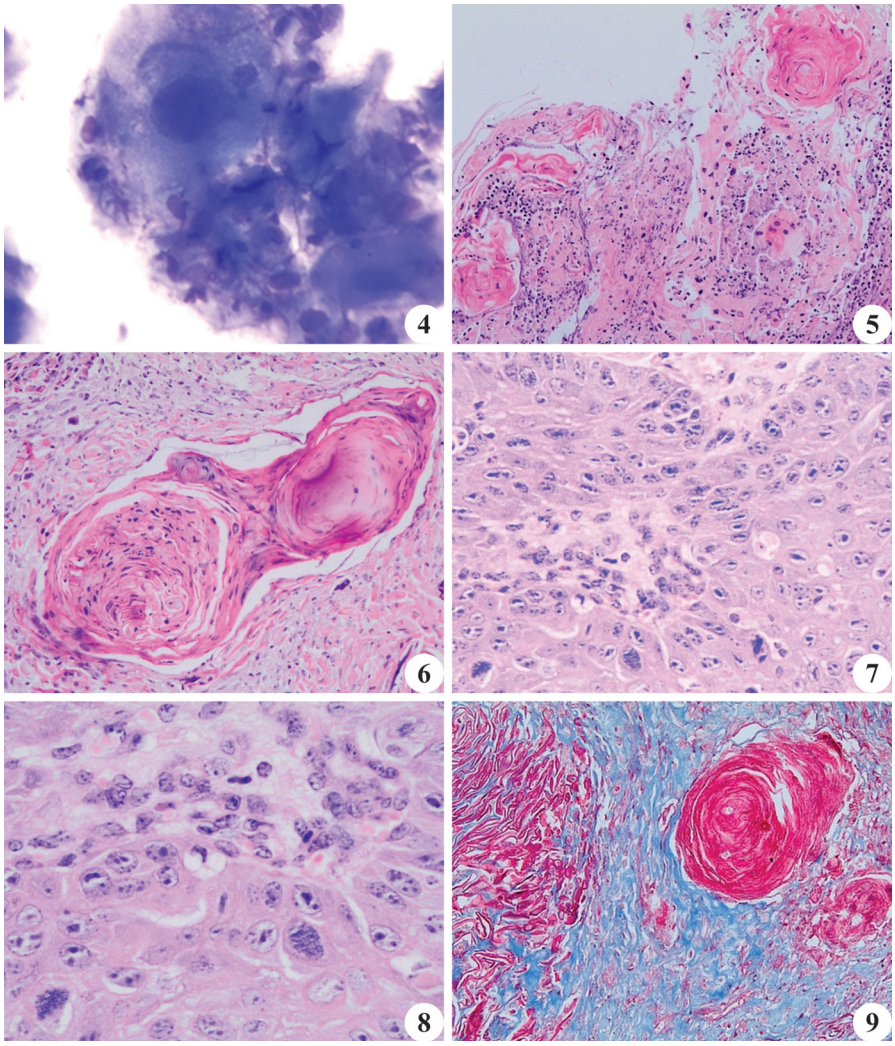

Fig. 4. Cluster of neoplastic squamous cells showing hyperchromasia, prominent anisokaryosis and anisocytosis along with inflammatory cells and keratin debris (Giemsa stain x1000); **Fig. 5.** Epithelial pearl or cell nests of proliferating neoplastic cells (H&E stain x100); **Fig. 6.** Epithelial pearl or cell nest of proliferating neoplastic cells surrounded by the fibrous connective tissue (H&E stain x200); **Fig. 7.** Proliferating neoplastic cells showing hyperchromasia, cellular pleomorphism, large vesicular nuclei, prominent and multiple nucleoli and frequent mitotic figures (H&E stain x400); **Fig. 8.** Proliferating neoplastic cells showing hyperchromasia, cellular pleomorphism, large vesicular nuclei, prominent and multiple nucleoli and frequent mitotic figures (H&E stain x1000); **Fig. 9.** Hyper keratinsed squamous epithelial cells taking reddish color, epithelial pearl or cell nest surrounded by blue stained connective tissue (Masson Trichrome Stain x200).

attributed by the stress in aged animal, with higher incidence in males (66.66%)[15].

Squamous cell carcinoma is a relatively more common and fatal neoplasm in cattle. The present case was a well differentiated SCC with lamellated keratin pearls and marked fibroplasia or desmoplasia in the deeper area of the neoplasm. The cytological and histopathological evaluation accompanied by special staining favored rapid diagnosis with the degree of cellular differentiation which was also of a good prognostic value to clinicians. For treatment of horn cancer amputation of horn alone or along with chemotherapy using Vincristine were on reports[15].

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Himachal Pradesh State Animal Husbandry Department and Deputy Director, Animal Health/Breeding, Distt. Una for providing necessary facilities for the present work.

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Indian J. Vet. Pathol., 48(1) : 78-80, 2024: DOI: 10.5958/0973-970X.2024.00013.0

CASE REPORT

Ovine rhinofacial pythiosis - A case report

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Received: 31.8.2023; Accepted: 31.10.2023

ABSTRACT

Pythiosis is an emerging infectious disease caused by the aquatic oomycete *Pythium insidiosum*, a fungal-like organism. The present report highlights the pathomorphological features of pythiosis recorded in the nasal cavity of a 2-year-old female non-descriptive sheep presented to the Veterinary Clinical Complex, Rajiv Gandhi Institute of Veterinary Education and Research, Puducherry, India. Clinical examination revealed serosanguineous nasal discharge from the left nostril and a growth which was intact with the maxillary bone. Incisional biopsy sample fixed in 10% neutral buffered formalin was processed by routine paraffin embedding technique and sections stained with H&E. Histopathological examination revealed a characteristic pyogranuloma with central amorphous eosinophilic area surrounded by neutrophils, degranulated eosinophils and karyorrhectic debris. Gomori methenamine silver staining revealed thin-walled, non-parallel and sparsely-septate hyphae with ballooning dilations. Based on the clinical signs, histopathology and histochemistry the case was diagnosed as ovine rhinofacial pythiosis.

Keywords: Clinical signs, histochemistry, histopathology, ovine rhinofacial pythiosis

Pythiosis is a potentially fatal infectious disease of animals and humans caused by *Pythium insidiosum* throughout the world. *Pythium insidiosum* is a fungus-like peronosporomycete (Oomycete) aquatic organism belonging to the kingdom Stramenopila phylum Oomycota, and order Pythiales (family *Pythiaceae*). It is believed that biflagellate mobile zoospores, which are the infective forms of *P. insidiosum* present in water, enter the body through skin injuries[1].

Among all reported pythiosis cases, the frequently affected hosts included horses followed by humans, dogs, cows, sheep, and cats. The infection is not contagious; no animal-animal or animal-human transmission has been reported so far[1]. Cases have also been reported in donkeys, mules, camels, bears, birds, goat, tiger, and jaguar. In animals, the disease has been mainly reported from the USA, Brazil, Australia, Colombia, Egypt, Venezuela, and Costa Rica. In India, the condition has been recorded in horses, shrimps and humans[2-4]. In sheep, the disease has been described in three forms, namely cutaneous, rhinofacial and digestive. Among them, the cutaneous and rhinofacial forms have been reported to cause 100% mortality and severe economic loss for the farmers[5]. Considering the importance of the disease and lack of reports of pythiosis in India, the present communication describes pathological features of ovine rhino facial pythiosis recorded in the clinical case.

A two-year-old non-descriptive ewe from a flock of twelve sheep, raised extensively was presented to the Veterinary Clinical Complex, Rajiv Gandhi Institute of Veterinary Education and Research, Puducherry, India with the history of slowly progressing unilateral swelling in the nasal cavity, nasal discharge and dyspnoea. After clinical examination, incisional biopsy was performed under local anaesthesia and tissue samples were collected. The tissues were fixed in 10% neutral buffered formalin, processed by routine paraffin embedding technique and 4-5 µm sections were stained with Haematoxylin and Eosin stain. Serial sections were stained by Gomori methenamine silver and Periodic acid Schiff as described previously[6].

How to cite this article : Avinash, S.V., Uma, S., Gurunathan, N., Poobitha, S., Lakkawar, A.W., Kumar, R. and Nair, M.G. 2024. Ovine rhinofacial pythiosis - A case report. Indian J. Vet. Pathol., 48(1) : 78-80.

Clinical examination revealed unilateral serosanguinous discharge and cauliflower-like growth protruding from the left nostril which was attached to the maxillary bone almost occluding the nasal passage (Figs. 1 and 2). Gross lesion of the present case was similar to those described in Sheep from Brazil[7]and Texas, USA[8], wherein facial deformity with intranasal growth partially occluding the nasal passage along with necrosis involving the nasal septum were also recorded. Ovine rhinofacial pythiosis, also known as "bull nose" is characterized by necrotizing rhinitis with marked enlargement and deformity of the nasal region, severe respiratory difficulty and bloody nasal discharge[9].

Histopathological examination revealed intact ciliated

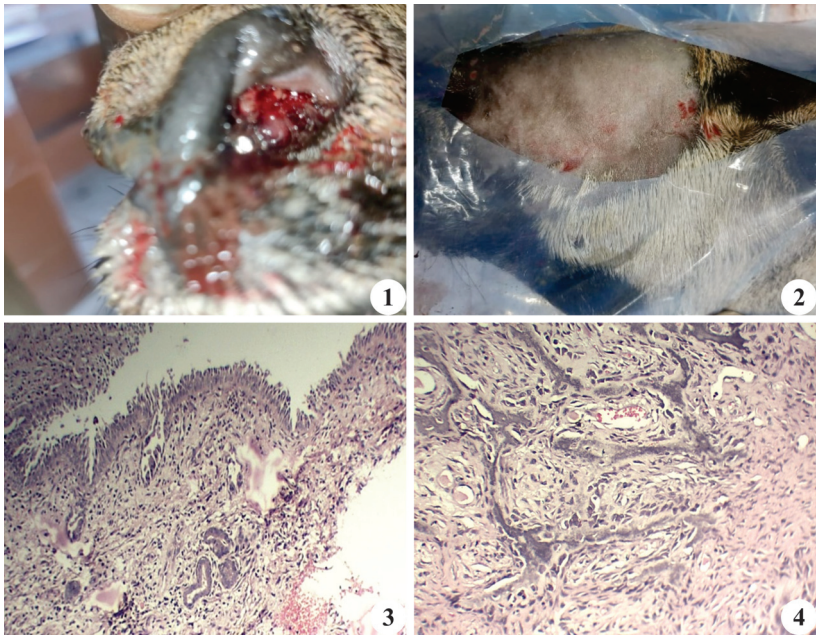

Fig. 1. Rhino facial swelling and serosanguinous discharge from the ulcerated lesion in the nasal cavity; **Fig. 2.** Sheep with rhino facial swelling; **Fig. 3.** Olfactory mucosa showing intact ciliated columnar epithelium with focal hyperplastic reaction (H&E 100x); **Fig. 4.** Nasal lesion showing rarefying osteitis (H&E 100X).

columnar epithelium with focal hyperplastic reaction (Fig. 3). The sub-mucosa revealed the presence of amorphous myxoid and rarefying osteitis amidst loose fibrous tissue proliferation (Fig. 4). Further, characteristic pyogranulomas with central amorphous eosinophilic area, surrounded by neutrophils, eosinophils, karyorrhectic debris (Splendore-Hoeppli reaction) were observed (Figs. 5 and 6). The microscopic features observed concurred with the reports of earlier workers[7,8,10]. *P. insidiosum* triggers in the infected host a T helper 2 [Th2] subset response with an inflammatory reaction composed mainly of eosinophils and mast cells. These cells degranulate around the hyphal elements of *P. insidiosum* leading to a Splendore-Hoeppli-like reaction[11].

In the present case, Periodic acid - Schiff staining failed to demonstrate the presence of fungal elements which could be due to the fact that PAS does not stain dead hyphae within the necrotic tissue (Splendore-Hoeppli material) or due to the lack of chitin, an aminopolysaccharide present in the cell wall of the fungus that is absent in *P. insidiosum*. However, Grocott methenamine silver staining revealed black coloured, thin-walled, non-parallel and sparsely-septate hyphae with dilatations (Fig. 7) and is similar to the findings of previous study[9].

Earlier studies have highlighted the involvement of other organs like lymph nodes and lungs in sheep through metastases[5,12] which were not recorded in the present study. Diagnosis of pythiosis can be made based on clinical signs as well as gross and microscopic lesions. Differential diagnosis however, in rhinofacial pythiosis be made from conidiobolomycosis that usually involves the rhinopharyngeal area[10].

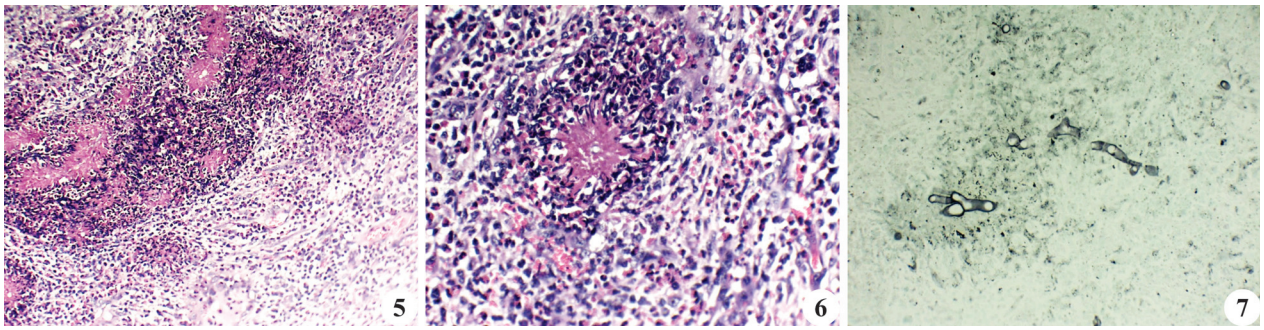

Fig. 5. Nasal lesion showing caseous necrotic area surrounded by neutrophils, eosinophils, karyorrhectic debris (Splendore-Hoeppli reaction) (H&E 40x); **Fig. 6.** Higher magnification of Fig. 5 showing the characteristic Splendore-Hoeppli reaction (H&E 400x); **Fig. 7.** Gomori methenamine silver stain highlights irregularly branching, nonparallel, thin-walled hyphae with bulbous dilation (GMS Stain, 400x).

The present case report describes rhino facial pythiosis in a sheep based on histopathology and histochemistry. The prognosis is poor if the disease is chronic and complete surgical excision is not possible. Although the incidence of the disease is low in sheep in India, an early diagnosis may help in evolving a control strategy.

ACKNOWLEDGEMENT

The authors are thankful to The Dean, Rajiv Gandhi Institute of Veterinary and Education and Research (RIVER), Puducherry for providing facilities to carry out this study.

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Indian J. Vet. Pathol., 48(1) : 81-84, 2024: DOI: 10.5958/0973-970X.2024.00014.2

CASE REPORT

Malignant cutaneous mast cell tumour in a dog : A case report

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Received: 16.8.2023; Accepted: 19.9.2023

ABSTRACT

Mast cell tumours (MCTs) are one of the commonest malignant skin cancer in dogs. In the present case, an adult male Labrador dog, aged about 6 years was presented in the veterinary clinical complex having a history of gradually developing swelling in the left thigh on medial aspect of tibia and femur for diagnosis and treatment. The swelling was progressive and has grown to a very big size in a period of about 7 months, involving whole thigh region. The affected area was focally necrosed, ulcerated and-bleeding with a tendency of itching. On gross examination, the swelling appeared as large a covering a wide area with severe inflammation, purulent myositis and cellulitis. On careful examination, few very palpable small firm masseswereobserved surrounding the primary lesion. Blood samples, fine needle aspirates from the affected site and ultrasound guided aspirates from inguinal lymph nodes were collected for cytological examination. Tissue samples were also collected in 10% formalin for Histopathological procedure. Ultrasonography was also performed to ascertain the extent of spread. The haematological examination showed increased neutrophil and eosinophils with mild anaemia. Cytological evaluation of fine-needle aspirates revealed many pleomorphic, highly granulated mast cells while the histopathological examination showed masses consisting of sheets of neoplastic round nucleated mast cells with granules in the cytoplasm along with eosinophilic and neutrophilic infiltrations. The purpose of this paper is to provide diagnostic procedure of mast cell tumours while ultrasonography can be a practical method for determining its stage to some extent.

Keywords: Cytopathology, dog, mast cell tumour, pruritus, skin swelling, ultrasonography

Mast cell tumours (MCTs) is frequently seen in some breeds of dogs and is extremely important in the field of veterinary oncology. It represents the third most common tumour subtype, and is the most common malignant skin tumour in dogs, making up approximately 20% of canine skin tumors[1]. Some dog breeds commonly are predisposed to MCT's are Boxers, Bull Terriers, Golden Retriever, Labrador Retriever, and Dachshund, while dog breeds like German Shepherd, Chihuahua, Poodle, Yorkshire Terrier and Cocker Spaniel are at a lower risk of MCT development[2]. Canine MCTs can be of different sizes,may be delimited, elevated, firm, soft, pruritic, ulcerated, erythematous with or without invasion of the subcutaneous tissue. The findings suggestthat about 50% of canine MCTs develop in the trunk, perineum, and inguino-genital regions, 40% occur in the limbs, and 10% in the head and neck[3]. Also, the metastatic movements frequently involvethe lymph nodes, skin, spleen, and liver and less frequently the lungs. These tumours exfoliate high numbers of typical cells containing large numbers of small, round, purple granules, making diagnosis relatively easy. Thus, cytopathological technique is themost routinely used, fast and cost effective method used to diagnose MCTs. Ultrasonography and computed tomography (CT) are imaging technique that has been used more frequently in recent years as a more sensitive tool in identifying metastatic lesions in different neoplasms[4].

MCTs have variable clinical presentation, since their biological behaviour is variable. In general,when MCTs are well-differentiated, they present a milder behaviour. In contrast, less-differentiated tumours have a more aggressive behaviour. In the current case, several clinical signs like rapid growth, pruritus, severe localised inflammation and infiltrative reactions, ulcerations, poor demarcation from adjacent tissues and satellite nodules in the affected dog was suggestive of MCTs exhibiting aggressive behaviour.

How to cite this article : Kumar, S., Tiwary, R., Gopal, M.A., Patel, R. and Bhagat, P.K. 2024. Malignant cutaneous mast cell tumour in a dog : A case report. Indian J. Vet. Pathol., 48(1) : 81-84.

An adult male Labrador dog aged about 6 years was presented in the veterinary clinical complex with the history of a small gradually developing swelling in the left l thigh on medial aspect of tibia and femur for the last about 7 months with severe and generalized pruritus. The dog showed reduced appetite, vomiting tendency, weight loss and apathy. The lesion was progressive and with time, it has involved whole thigh region. The dog was being treated with different antibiotics but no appreciable improvement was noticed. As per history, previous symptomatic treatments like anti-inflammatory, anti-histamine, antibiotics with suitable topical

gel application on the concerned area was given to the dog with no substantial improvement. Also, acaricidal treatment was provided to rule out any parasitic infestation. All these efforts lead to temporarily relief only with mild reduction insize of growth and pruritus with increase in appetite. With due course, the limb lesions were worsening, spreading onwards and not responding to treatment anymore and the animal remained apathetic with lot of sufferings.

Physical examinations revealed apathetic dog with deteriorating body conditions with slight rise in body temperature. Dermatological examination showed that the affected area was markedly enlarged, firm and the surface was ulcerated, bleeding, necrosed and covered with purulent, haemorrhagic discharge. The swelling appear to be covering a wide area with severe inflammation including purulent myositis and cellulitis on the medial aspect of leftthigh (Fig. 1). There was alopecia and scaling with erosions and ulcers observed on the affected area. On careful examination, there was few palpable small firm masses found surrounding the primary lesions. Blood samples, skin scrapings, touch smear, fine needle aspirates from site and ultrasound guidedaspirates from adjoining inguinal lymph nodes were collected for cytological examination (Fig. 2). The tissue samples were collected for further histopathological procedure and preserved in 10% formalin. Ultrasonography was also performed to ascertain the extent of spread.

The haematological examination showed increase in number of neutrophils (76%) and eosinophils (10%) with marked leucocytosis ($16\times10^3/mm^3$). The reduced value of haemoglobin (10.5 gm%) suggested mild anaemia. The biochemical values were within normal limits however,there was mild increase in the values of aspartate aminotransferase (65IU/L) and Alanine transaminase (145IU/L), suggesting mild liver disorders.

Ultrasound findings revealed that the swelling had poorly distinct margin and anechoic to hypoechoic area on affected muscle part (Fig. 3) while hypoechoic area on inguinal lymph node (Fig. 4). Also there was a very thick diffuse hyper-echogenic subcutaneous tissue with some fluid filled cavities on the affected part of the limb. Abdominal ultrasound showed increased size of lymph nodes with few hyperechoic foci, moderate hepatomegaly while other organs appeared almost normal.

Smears collected were stained with Giemsa stain. Cytological examination of the touch smear from necrotic areas showed mainly necrotic cells, degenerated neutrophils (pus cells), eosinophilic and neutrophilic inflammation with concurrent presence of different

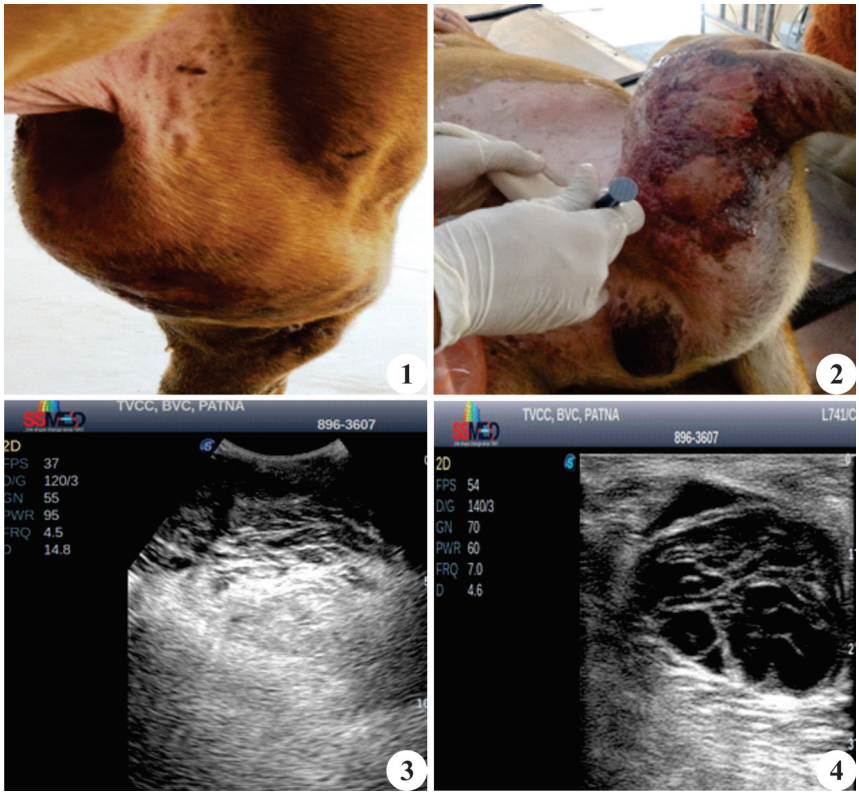

Fig. 1. Severe swelling in the affected left limb of dog; **Fig. 2.** Ultrasonography guided collection of aspirates from the inguinal lymph node; **Fig. 3.** Anechoic to hypoechoic area on affected muscle part of the dog; **Fig. 4.** Hypoechoic area on inguinal lymph node of the affected dog.

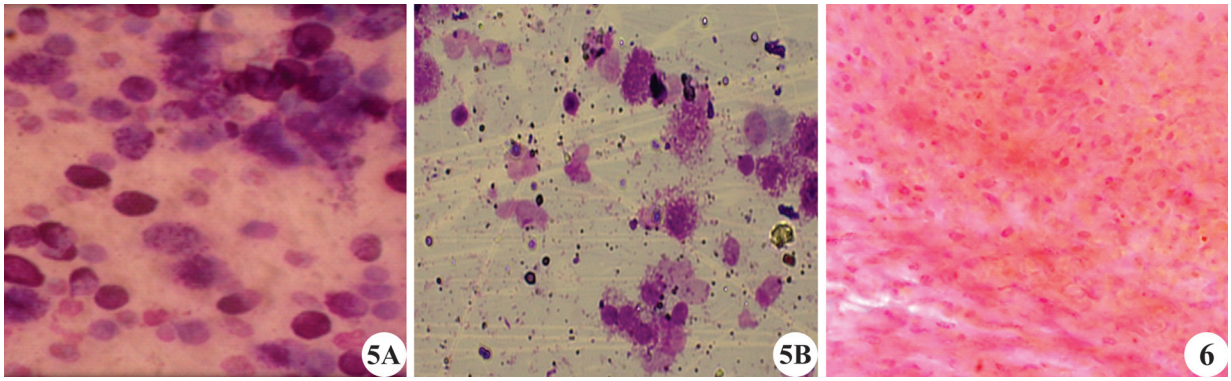

Fig. 5A and B. Pleomorphic highly granulated mast cells with some degranulation; Giemsa stain; **Fig. 6.** Pleomorphic neoplastic mast cells (H&E ×10).

forms of bacterial population. Cytological evaluation of fine-needle aspirates comprised many pleomorphic mast cells with highly granulated cytoplasm (Fig. 5A and B). Some of the mast cells were de-granulated which might have been occurred during collection of aspirates and smearing it. Marked cellular atypia was observed within the mast cell population thus including anisokaryosis, anisocytosis, hyperchromatism, prominent nucleolation, bizarre nuclei and good number of mitotic figures.

The histopathology was done by paraffin embedding technique and sections of 5µ were cut and routinely stained with H&E stain. The microscopic examination of stained slide revealed moderately pleomorphic neoplastic mast cells (anisokaryosis and anisocytosis), with round and pleomorphic nuclei having intracytoplasmic granulation of varying sizes which have extended into the subcutaneous and muscular tissue with discrete areas of edema, necrosis, and collagen hyalinization (Fig. 6). On an average one to two mitosis figures per high power field were also visible.

Based on the clinical behaviour and cyto-histopathological findings, the case was diagnosed as malignant mast cell tumour which was well authenticated by ultrasonography findings. The grading system proposed by Patnaik *et al*.[5], is the most commonly used system for cutaneous histological classification of MCTs, and divides tumours into grades 1, 2, and 3. The findings suggests that the present case can be concluded to be a case of grade 2 mast cell tumour. However, more elaborative work was needed to be done for confirmation of its grade and thereafter, suggest treatment regime. Staining with toluidine blue and immunohistochemistry would have thrown better insights. Combining Ki-67 expression with toluidine blue staining is useful in differentiating MCTs from other lesions such as eosinophilic granuloma[6].

Mast cell tumours of the skin can occur anywhere on the body and there is significant variation in their biological behaviour. They can appear as a raised lump or just under the skin. Similarly they may be red, ulcerated, or swollen. Degranulation of the histamine causes inflammatory pruritus and subsequent swelling of the surrounding tissue. The etiology of MCT has not been completely elucidated. However, it is postulated that the influence of chronic inflammation in the skin and exposure to irritating compound (allergens) can be inciting causes. Furthermore, the presence of mutations in the c-KIT gene (*KIT*) has been related to tumour development in MCT cases. This gene encodes a receptor tyrosine kinase that binds stem cell factor (SCF) in canine mast cells. Mutations drive uncontrolled cell survival and proliferation, which is related to MCT development and progression[7].

The metastatic potential of MCTs varies according to its histopathological classification. In well-differentiated mast cell tumours, metastasis occurs in less than 10% cases;in moderately differentiated neoplasms, metastasis occurs in 5% to 22% cases and up to 55% to 95% metastasis occurs in poorly differentiated cases. Metastases occur mainly on regional lymph nodes and may later affect the spleen, liver, and other organs. Patients with mast cell tumours of any degree and who have regional lymph node involvement in general have a poorer prognosis[8]. The main therapeutic measures indicated in the cases of canine MCT is surgical excision, which may be suggested whenever possible[9].

Fine-needle cytology is the basis of MCT diagnostic investigation and is often tried whenever there is the initial doubt. Cytopathology alone can help arriving the correct diagnosis in 92 to 96% of cases. Tissue collection is usually performed for further histopathology and diagnosis. Romanowski technique are effective for staining mast cell granules. In conclusive cases, where mast cell granules staining does not occur, the use of special stains, such as Giemsa and toluidine blue, is recommended[10].

In conclusion, canine MCTs are one of the common neoplasms which can present a high degree of malignancy. These tumour can be accompanied by fever,

cellulitis, myositis and diffuse pruritus. Diagnosis can be done based on clinical behaviour, cytology and histopathological findings, however special staining and immunohistochemistry can be done to further access the biological behaviour of the neoplasm. Ultrasonography can be helpful in determining the spread and thus staging of the tumour.

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Indian J. Vet. Pathol., 48(1) : 85-87, 2024: DOI: 10.5958/0973-970X.2024.00015.4

CASE REPORT

Pathological analysis of renal failure and its repercussions in canine : A case report

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Received: 22.8.2023; Accepted: 12.9.2023

ABSTRACT

A pathological investigation was undertaken on a 7-year-old male American bully dog, brought to the Department of Veterinary Pathology, Dr G.C. Negi COVAS, Palampur to elucidate the possible causes of death, with the history of vomiting, polyuria, and anorexia. Serum biochemical estimation has revealed elevated levels of blood urea nitrogen (BUN), creatinine and glucose. On necropsy, the visceral organs were found pale, along with enlarged and deformed kidneys. Microscopically, the kidneys showed cellular swelling of the tubular epithelium, dilatation of the tubules, desquamation of the tubular epithelium, and presence of eosinophilic material along with mild fibrosis around the degenerated tubules.

Keywords: Chronic renal failure, dog, kidneys

Chronic renal failure (CRF) or chronic kidney disease (CKD) represents the loss of functional renal tissue brought on by an illness that has advanced for more than two months[1]. CKD is more frequently observed in geriatric dogs and cats[2]. The loss of nephrons and decreased blood flow to the kidneys with advancing age attribute to an increased risk of renal failure in older dogs. The researchers have reported the highest incidence of renal failure in older dogs (49.58% in >8 years), followed by middle age (35.17% in >4-8 years), and the least in younger dogs (15.25%, <4 years of age)[3]. The progression of regular wear and tear caused by cellular senescence is held responsible for the gradual development of chronic kidney disease (CKD) among older persons[4]. If CKD is acquired once, it becomes irreparable and causes concerns about reduced renal health[5]. Increased levels of phosphorus, salt, and high-protein diets in both cats and people are also thought to contribute to the development of CKD[6].

An American bully was presented to the Department of Veterinary Pathology, DGCN COVAS, Palampur, for necropsy examination. A systematic post-mortem examination comprised a detailed external and internal examination of organs. Gross lesions were throughly examined and recorded. The animal passed away while receiving treatment at the DGCN College of Veterinary and Animal Sciences, Palampur. During the necropsy examination representative tissue samples were taken for histopathological examination in 10% neutral buffered formalin (NBF) solution.

After 48 hours of fixation, tissues were processed and stained with haematoxylin and eosin (H&E) stain in accordance with routine histopathological technique[7].

In the serum biochemical estimation of BUN and creatinine, the values were found to be 95.49 mg/dl and 1.171 mg/dl, respectively. The altered values of BUN and creatinine in serum with reference ranges are presented in Table 1. Gross pathological examination has revealed the presence of subcutaneous oedematous fluid all over the body, or anasarca (Fig. 1), and straw-coloured ascitic fluid in the abdominal cavity (Fig. 2). The liver was slightly enlarged and showed diffuse congestion with fibrin. Kidneys showed irregularly elevated blebs on the surface; the renal capsule was tightly adhered to the surface; fluid-filled cavitations were present on the medulla; and the cortex was diminished from the borders due to the disproportionate surface of the kidney (Fig. 3). The spleen was slightly enlarged. The lumen of the intestine exhibited diffuse haemorrhages with catarrhal exudate in the lumen. Histopathological examination of kidneys depicted severe degenerative changes characterised by cellular swelling of tubular epithelium, loss of brush borders, deterioration

How to cite this article : Bisen, H.K., Kumar, R., Joshi, G., Verma, A., Katoch, S., Bisht, E. and Asrani, R.K. 2024. Pathological analysis of renal failure and its repercussions in canine : A case report. Indian J. Vet. Pathol., 48(1) : 85-87.

Table 1. Serum biochemical analysis for Blood Urea Nitrogen (BUN) and Creatinine levels in dog.

Biochemical test	Result	Unit	Reference range
BUN	95.49	mg/dL	8-28
Creatinine	1.71	mg/dL	0.5-1.7

of tubular epithelium, and the presence of eosinophilic material. Peri-tubular fibrosis and cystic spaces were also documented in the cortical region, along with mononuclear cell (MNC) infiltration (Fig. 4). Liver section was found to show hydropic degeneration, fibrin deposition, engorged sinusoids, and infiltration of MNCs. Intestine showed mild congestion, desquamated mucosa with goblet cell hyperplasia, and infiltration of MNCs.

Some of the studies address the fact that in geriatric dogs, the episodes of renal failure may be worsened by pre-existing diabetes, bacterial pyelonephritis, amyloidiosis, polycystic kidneys, urolith or nephrolith formation, and renal tumours[8]. It has been assumed that, in particular, pathological alterations in relation to hypoxic insults are more frequent in the cortical area of the kidneys as compared with the medulla[4]. In the present study, the elevated levels of BUN and creatinine in serum indicated renal damage[9]. In one of the studies, it was reported that on gross examination, the kidneys were pale, enlarged, and misshapen with an adherent renal capsule[10]. The histological picture has depicted marked tubular degeneration, dilated tubules, and the presence of fibrous tissue in the renal parenchyma. These gross and microscopic findings in the kidneys of a dog have a direct correlation with the observations of our study. The fibrous tissue deposition along with degenerative changes in the kidneys of dog in the present study are in concordance with the previous study concluded by the researchers[11]. It is widely accepted that the reversal of compromised renal function can only be achieved if the tubular basement membrane is intact. Furthermore, more complex hypoxic insults causing damage to the tubular basement membrane support the development of more severe chronic changes[6]. The above-mentioned notions provide widely accepted support for the study conducted by us, as the chronic nephritis recorded in the above case has quite a similar pattern.

In comparison to the general population, patients with CKD have a greater rate of mortality[12]. Various epidemiologic studies have certified that even a mild elevation in serum creatinine level is associated with an increased rate of mortality from any cause[13]. Chronic kidney damage may be a substantial risk factor for mortality regardless of comorbid conditions such as diabetes, dietary factors, and hypertension. Further associated conditions and disorders include morbidity and mortality from cardiovascular problems (such as angina, left ventricular hypertrophy [LVH], and

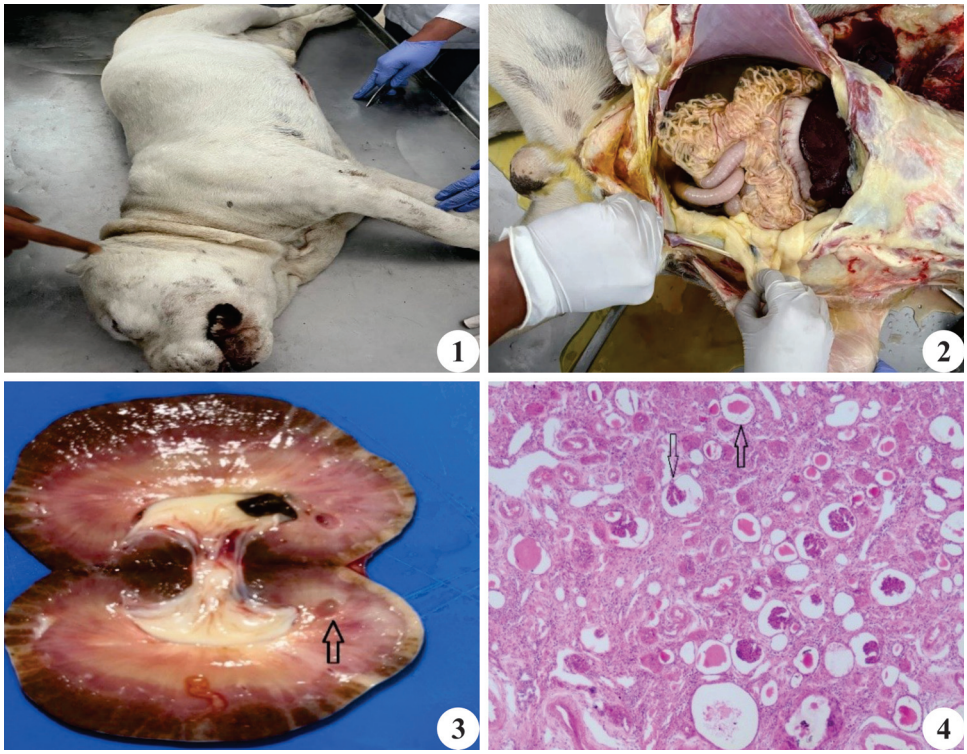

Fig. 1. Generalized oedema/anasarca; **Fig. 2.** Yellow coloured ascitic fluid with fibrinous threads present in the abdominal cavity; **Fig. 3.** Kidney showing pale discolouration along with cavitations in medullary area (arrow); **Fig. 4.** Kidney section depicting glomerular atrophy (thin arrow) with cystic tubules (thick arrow) and fibrous tissue deposition (H&E x400).

Table 2. Repercussions associated with chronic renal damage in dog.

Test	Result	Unit	Reference range
Red Blood Cell	1.03 L	Milln/µl	5.5-8.5
Haemoglobin	3.0 L	g/dL	12-18
Platelet	0.066 L	10^3/µl	2-5 L
Alkaline Phosphatase	404.51	U/L	20-150
Glucose	144.56	mg/dL	76-119

increasing heart failure) that are increased by anaemia, which frequently coexists with advancing CKD[14] as represented in Table 2. Disorders of the bones and minerals that are linked to CKD include anomalies in mineral and bone metabolism[15,16]. Reduced renal phosphate excretion may be attributed to a number of factors. In addition to GFR, the renal tubules additionally serve a role in phosphate reabsorption; damage to the tubules can lead to reduced phosphate excretion. The kidney plays an integral role in phosphate homeostasis by means of the hormone fibroblast growth factor 23 (FGF23), which has the ability to increase phosphate excretion. In Table 2 biochemical test, an increased ALP level was found. In all stages of chronic kidney disease, diabetes is linked to negative outcomes[17].

Chronic kidney damage (CKD) in canines can be of multiple aetiologies, primary ones like dietary imbalance leading to strain on kidneys in the long run, leading to irreversible damage leading to consequences such as renal damage and other deleterious effects like anaemia, as an insult to the kidney will result in diminishing ability to produce erythropoietin, a hormone vital for RBC production; other complications like reduced phosphate will result in hyperphosphatemia and can result in renal osteodystrophy; additionally, cardiovascular risk can be fatal.

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Indian J. Vet. Pathol., 48(1) : 88-90, 2024: DOI: 10.5958/0973-970X.2024.00016.6

CASE REPORT

Concurrent infection of Sarcoptes mange with Staphylococcosis in a rabbit - A case report

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Received: 12.8.2023; Accepted: 20.9.2023

ABSTRACT

In order to identify the cause of death, a carcass of a New Zealand white male adult rabbit presented to Department of Veterinary Pathology, GADVASU, Ludhiana with the history of lethargy, emaciation and multiple skin lesions. A detailed investigation of thecarcass revealed areas of dry crusty lesions in ears, subcutaneous abscess on abdomen and pododermatitis. Parasitological examination of skin scrapings taken from the carcass confirmed presence of Sarcoptesscabiei. Microscopic examination revealed erupted epidermis along with embedded parasites and eggs.Samples of the skin lesions were taken and characterized microbiologically by culture. Bacterial colonies of *Staphylococcus aureus* were identified by MALDI TOF.

Keywords: Abscess, pododermatitis, *Sacrcoptes*, *Staphylococcus*

How to cite this article : Masand, R., Jadhao, A., Kamra, R., Singh, S. and Brar, A.P.S. 2024. Concurrent infection of Sarcoptes mange with Staphylococcosis in a rabbit - A case report. Indian J. Vet. Pathol., 48(1) : 88-90.

Sarcoptic mange is most fatal and contagious parasitic infestation of rabbits caused by scavenging mite, Sarcoptesscabiei of family Sarcoptidae, manifested by hyperkeratosis, alopecia, seborrhoea and severe pruritis. It is one of the most common diseases seen in rabbits leading to huge economical losses to the rabbit breeders[1]. The lesions are usually dry crusty areas observed on ears, nose, feet and perineal region[2,3]. Poor hygiene and overcrowding are the two most critical predisposing factors for scabies[4]. This ectoparasite, also known as an itch mite, burrows into the epidermis of the skin and causes scabies in humans and mange in animals[5].

Staphylococcosis is bacterial disease caused by *Staphylococcus aureus* in rabbitries, which are characterised by pododermatitis, mastitis, subcutaneous abscesses and septicaemia[6]. Occasionally, abscesses are also observedin lungs, liver and uterus, which may lead to infertility, poor production and even death[7]. *Sarcoptes scabiei* may invade skin barriers and excrete certain molecules, which inhibit innate immunity of host promoting the infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes*[6].

A carcass of a New Zealand white male adult rabbit brought to Department of Veterinary Pathology, GADVASU Ludhiana with the history of emaciation, lethargy and multiple skin lesions for necropsy examination. A thorough external examination of the carcasswas performed for the presence of ectoparasites or injury. The systematic necropsy examinationwas performed and gross lesions were recorded. Tissue samples were collected in 10% formalin, processed, sectioned and stained with haematoxylin and eosin stain as per the standard protocols[8]. Skin scrapings were collected from affected areas, were placed in a petridish, and treated with 10% KOH (Potassium hydroxide) solution. The mixture was stirred, centrifuged and supernatant discarded. A few drops of fluid on a slide were used to examine the mites under a microscope.

Samples from the skin lesions collected in sterile swab for bacterial isolation and were inoculated on nutrient agar and incubated at 37^{O}C for 24 hrs. The colonies were picked up for gram's staining. Cytosmear were Cultural growth employed to MALDI TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) for further confirmation.

Gross examination of the carcass revealed crusted irregular raised dried scabs in ears, pododermatitis and subcutaneous abscesses throughout the carcass (Figs. 1 and 2). Rounding of the heart was observed. There was catarrhal exudate in the stomach and intestine. Stomach mucosa also revealed congestion and few types of erosion.

Adult *Sarcoptes scabiei* parasites were detected in rabbit skin scrapings (Fig. 3). The adult mite have four pairs of short legs with oval, ventrally flattened and dorsally convex tortoise-like bodies and cuticular spines on the dorsal side. Adult female mite was having rounded body,

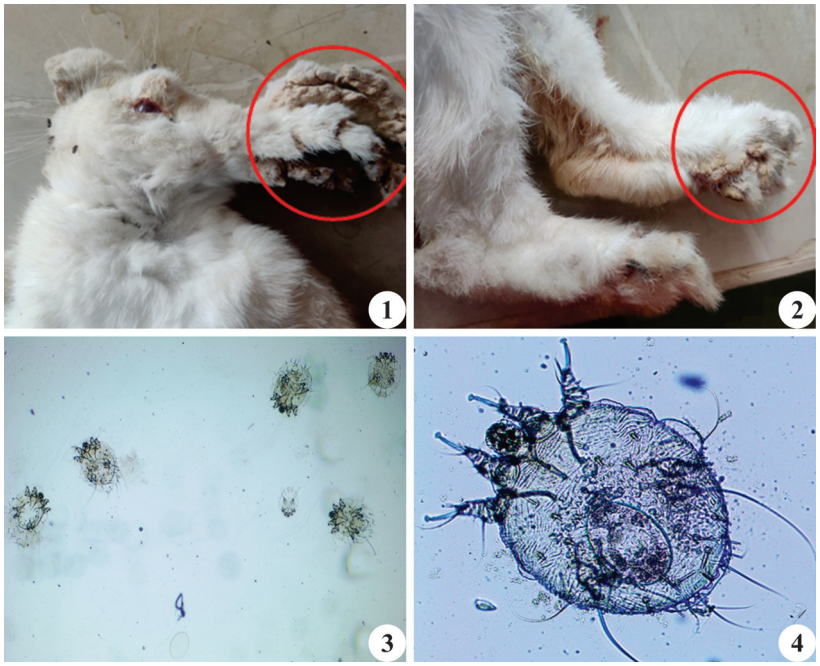

Fig. 1. Crusted irregular raised dried scabs (circle); **Fig. 2.** Pododermatitis (circle); **Fig. 3.** Photomicrograph of the sarcoptes mite; **Fig. 4.** Ventral view of adult female mite with rounded body, shorter legs and anterior end with suckers.

shorter legs and anterior end with suckers (Fig. 4).

Histological examination of the skin lesions revealed diffuse irregular thickening of epidermis, hyperplasia of stratum spinosum along withformation of rete ridges and infiltration of mononuclear cells and cellular debris were seen in the epidermis. Parakeratotic hyperkeratosis of stratum corneum with serocellular crust formation was also evident (Fig. 5). Multiple cross sections of oval to irregular embedded mite were observed in stratum corneum, which was recognised by a chitinous

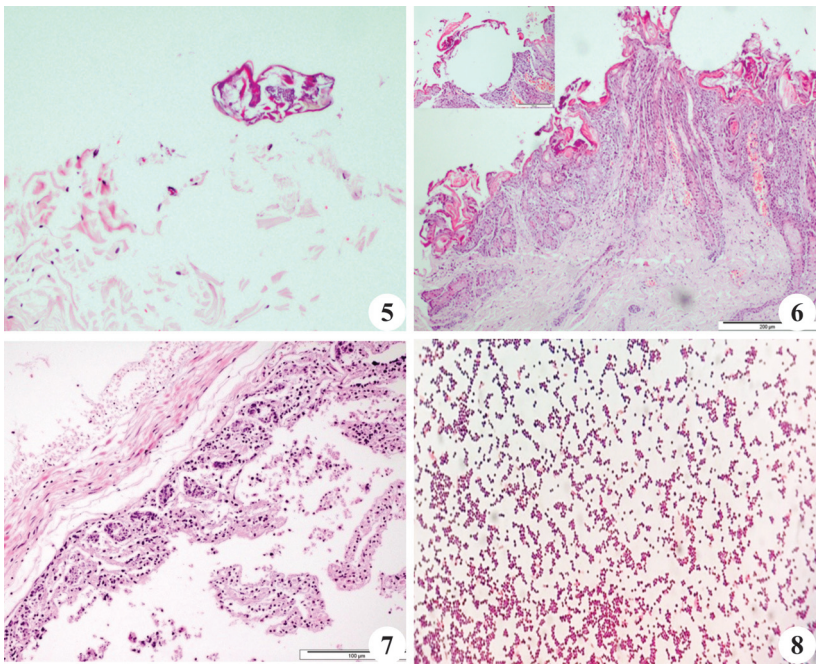

Fig. 5. Skin: Cross section of the oval to irregular arthropod parasite characterized by a chitinous cuticle, striated muscle, and a body cavity (*Sarcoptes sp.*) within the stratum corneum (H&E, 20X); **Fig. 6.** Microphotograph of skin showing presence of mites in burrows in the epidermis; serocellular and eosinophilic crust with mild oedema along with hyperplasia of stratum spinosum, rete papillae and mononuclear inflammatory cells infiltration (H&E, 20X); **Fig. 7.** Intestinal villi sloughed off (H&E, 20X); **Fig. 8.** *Staphylococcus aureus* in cluster, chains, and singles arrangements isolated from affected skin of rabbit (Gram stain, 10X).

cuticle, striated muscle and body cavity (Fig. 6). Lungs showed congestion, haemorrhages and bullae formation. Intestinal villi were sloughed off at places (Fig. 7). Glomerular blood vessels were congested and renal tubules were having pale eosinophilic proteinous mass. Liver revealed sinusoidal congestion and mild infiltration of mononuclear cells in periportal areas. Degeneration of hepatocytes was also evident.

On gram staining, gram-positive cocciwere observed which were present in singles, clusters and chains, resembling *Staphylococcus spp.* (Fig. 8). Bacterial culture of *Staphylococcus aureus* was confirmed by using MALDI TOF.

Ear mite and mange infestations have been described as severe skin conditions in both young and adult rabbits. The presence or lack of pruritis, the shape of the mite, and the distribution of lesions characterise *S. scabiei* mange in rabbits[9]. The presence of mites under a microscope, as well as distinctive skin lesions in various body areas, confirms the presence of sarcoptic mange in rabbits. Burrowing activity and mechanical damage induced by the parasites during excavation, irritating action of their secretion, allergic reactions to some of their extracellular products, and specifically release of interleukin-I leads to pathological lesions[10].

On the basis of gross, histopathology, parasitology and microbiological findings, it was confirmed to be concurrent infection of sarcoptes mange and staphylococcus aureus in rabbit. Since it is highly contagious, steps should be taken to prevent it and otherwise may leads to heavy economic losses to rabbitry farms.

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Indian J. Vet. Pathol., 48(1) : 91-94, 2024: DOI: 10.5958/0973-970X.2024.00017.8

CASE REPORT

A reactive systemic amyloidosis with fibrosis in a Vigova duck : A case report

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Received: 11.8.2023; Accepted: 31.10.2023

ABSTRACT

A 2-year-old female Vigova duck weighing 3.2 kg was presented to the Department of Veterinary Pathology for a postmortem with an enlarged abdomen and swelling of the plantar aspect of the feet. Gross examination revealed a severely enlarged pale pink liver weighing 440 g, occupying almost the entire length of the abdominal cavity. Microscopically, the liver revealed extensive deposition of homogenous, eosinophilic material in the hepatic parenchyma, replacing the hepatocytes, along with infiltration of mononuclear cells. The homogenous, eosinophilic materials filled and expanded the interstitium of the spleen, kidney, intestine, and ovary, in addition to the liver. The homogenous, eosinophilic material was confirmed to be amyloid with Congo red stain. Further, Masson's trichrome staining revealed the presence of fibrosis along with amyloid deposition. Culture from the necrosed region of the foot revealed bunches of Gram-positive cocci, indicating *Staphylococcus* organism. Based on gross, histopathological, and microbiological examination, the present case was diagnosed as systemic amyloidosis with fibrosis secondary to ulcerative pododermatitis in a Vigova duck.

Keywords: Congo red, duck, masson's trichrome, pododermatitis, *Staphylococcus*

How to cite this article : Nair, A.P., Krishna, B.D., Pradeep, M., Palekkodan, H., Madhanraj, N., Rajasekhar, R., Anoopraj, R. and George, A.J. 2024. A reactive systemic amyloidosis with fibrosis in a Vigova duck : A case report. Indian J. Vet. Pathol., 48(1) : 91-94.

A pathologic condition called "avian amyloidosis" develops in domestic ducks as a result of persistent inflammation and infectious or cancerous situations[1]. The condition is characterized by the extracellular deposition of an insoluble substance called amyloid within various tissues and organs of the body[1]. Amyloid is an eosinophilic homogenous material derived from the protein precursors with a characteristic fibrillar pattern[2]. The most common type of amyloid in birds is found to be AA-type, derived from an acute-phase reactant serum amyloid A (SAA)[3]. Although the pathogenesis of avian amyloidosis was not clearly explored, some predisposing factors, such as chronic inflammation and infections, could lead to the accumulation of amyloid in various tissues[3]. Diagnosis of amyloidosis occurs rarely in antemortem due to nonspecific clinical signs. Hence, histopathological studies are required for its confirmation. The present article describes the gross and histopathological characteristics of amyloidosis with fibrosis secondary to ulcerative pododermatitis in a Vigova duck.

A 2-year-old Vigova duck that had been raised under an intensive system and weighed 3.2 kg was brought in for a postmortem examination to the Department of Veterinary Pathology at the College of Veterinary and Animal Sciences in Pookode. The duck had a history of lameness, lethargy, decreased egg production, foot swelling, and an enlarged abdomen. External examination of the carcass revealed an enlarged abdomen with ventral lacerations and swelling on the plantar region of the feet, thickening of the skin, and a centrally swollen necrotizing crater-forming ulcer. At necropsy, a severely enlarged, pale pink, firm liver occupied almost the entire length of the abdominal cavity. The surface of the liver was pale pink, smooth, and glistening, with focal capsular thickening and rounded edges. The liver was 440 g in weight and its caudal end was well past the sternum (Fig. 1A). Moderate splenomegaly, diffuse gastrointestinal serosal congestion, and engorged mesenteric blood vessels were among the other findings. There were hemorrhagic mucoid materials inside the intestinal lumen. The spleen and kidney were enlarged and congested. The ovary was moderate to severely atrophied and hyperaemic (Fig. 1B). In the leg (Fig. 1C), the plantar swelling's opening revealed that there was gooey pus present in the cavity (Fig. 1D). For histopathological analysis, tissue samples were gathered and preserved in 10% neutral buffered formalin. The tissues that had been treated with formalin were prepared for paraffin embedding. Hematoxylin and eosin were

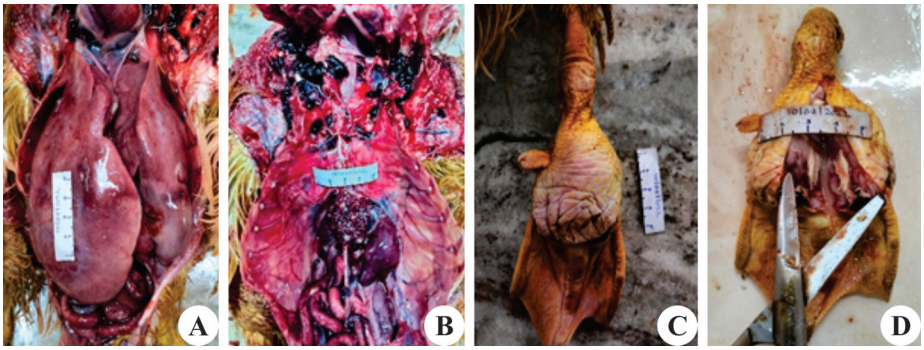

Fig. 1A. Severely enlarged pale pink, firm liver with focal capsular thickening occupying the entire abdominal cavity. **B.** Atrophied ovarian follicles and diffusely enlarged congested spleen. **C.** Diffuse plantar swelling with thickening of skin. **D.** Presence of pus in a cut section of plantar swelling.

used to stain sections that were 5 µm thick. Congo red and Masson's trichrome were used to further stain serial tissue sections. Tissue from plantar foot were stained with Brown and Brenn method[4].

Microscopically, in the liver, moderate to severe deposition of eosinophilic amorphous substances in the 'space of Disse', periportal, and perivascular areas with effacement of hepatocytes was noticed. Large amounts of amorphous pink deposits obliterated the sinusoidal spaces, leading to compression atrophy and degenerative changes in hepatocytes (Fig. 2A). Islands of atrophied hepatocytes with diffuse infiltration of mononuclear cells, such as plasma cells, and lymphocytes with fewer heterophils were present in the hepatic parenchyma. The amorphous deposition of liver turned orange to red when stained with Congo red, confirming that it was amyloid deposition[4].

Amyloid accumulation in the spleen led to hyalinization of the walls of ellipsoids and penicillar capillaries. (Fig. 2B). Also, multifocal nodular deposition was noticed in the red pulp region of the spleen, along with disruption and effacement of the ellipsoidal and periellipsoidal white pulp by amorphous eosinophilic substances. Histopathologically, the ovary showed congestion and thickening of the blood vessel wall in addition to atrophied ovarian follicles. A moderate amount of amyloid deposition was noticed focally in the interstitium and around the wall of the thickened

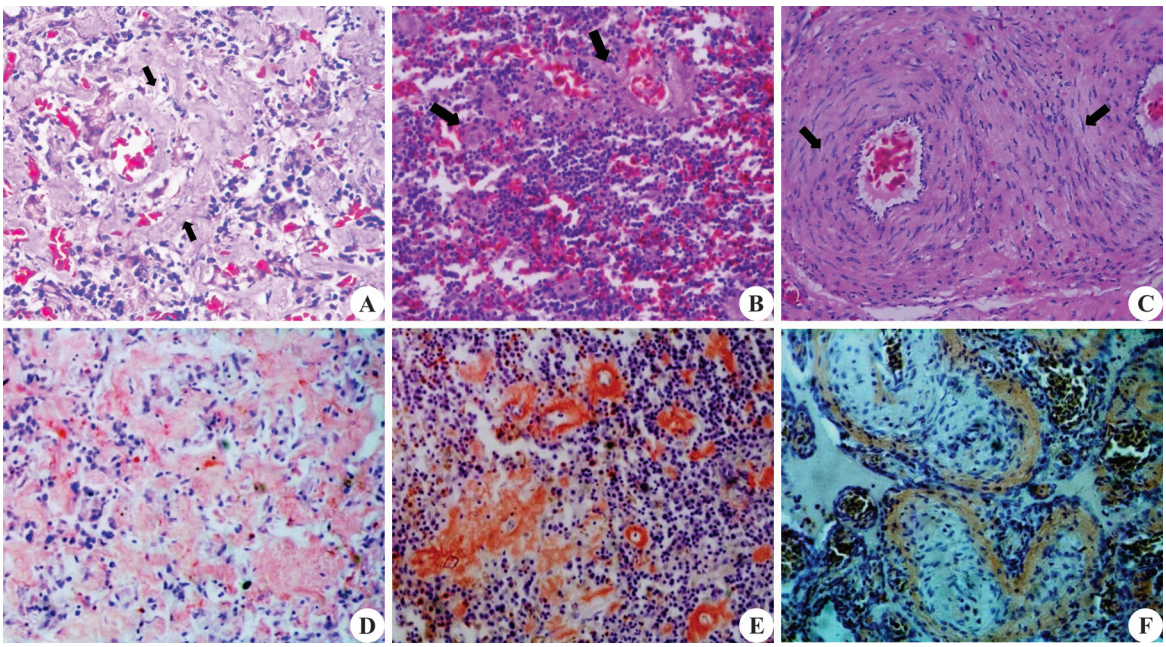

Fig. 2A. Severe diffuse deposition of amorphous pink substance (arrow) in the sinusoidal space with atrophy of hepatocytes and infiltration of mononuclear cells (Liver, H&E x400). **B.** Diffuse multifocal nodular amorphous deposition in red pulp. Disruption and effacement of ellipsoidal area of white pulp by pink amorphous material (arrow) (Spleen, H&E x400). **C.** Thickening of blood vessel wall and eosinophilic deposition (arrow) (Ovary, H&E x400). **D.** Amyloid deposition in space of Disse stained orange (Liver, Congo red x400). **E.** Multifocal nodular amyloid deposit in the red pulp, periarteriolar area (Spleen, Congo red x400). **F.** Moderate deposition of amyloid around thickened blood vessels (Ovary, Congo red x400).

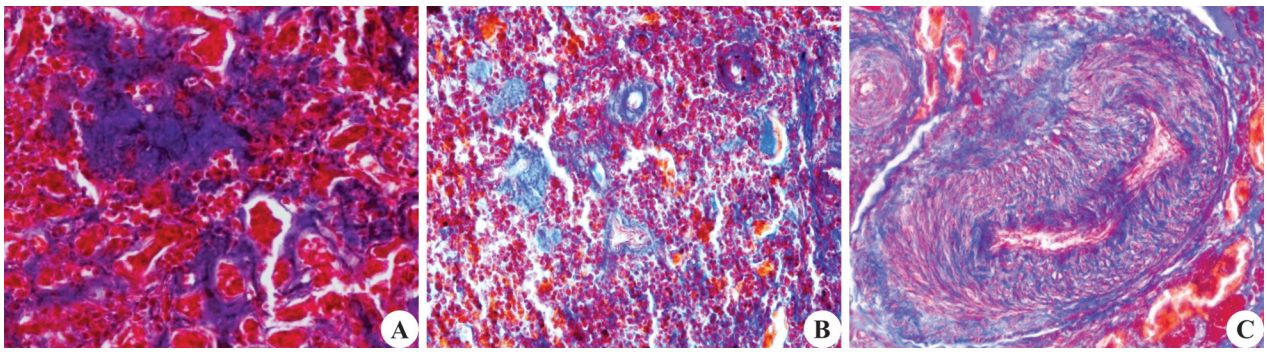

Fig. 3A. Deposits of amyloid bound without collagen fibres in the sinusoidal space stained blue and amyloid bounded collagen fibres-stained red (Liver, Masson's Trichrome x400). **B.** Amyloid deposits in periarteriolar area stained blue (Spleen, Masson's Trichrome x400). **C.** Amyloid deposition in thickened blood vessels stained blue (Ovary, Masson's Trichrome x400).

blood vessels (Fig. 2F). In kidneys, a mild amount of amyloid deposition was found around the walls of the renal blood vessels and tubular basement membrane. Desquamation of tubular epithelium and degenerative changes of renal tubules with infiltration of mononuclear cells were evident. Diffuse, moderate amyloid deposits were present in the lamina propria of the duodenum with severely congested blood vessels and infiltration of mononuclear cells such as plasma cells and lymphocytes. Further staining with Masson's trichrome revealed that the amyloid-free collagen fibre stained blue while the amyloid-bound collagen fibre stained red in other organs (Fig. 3A-C). In addition to amyloid deposition, the presence of collagen deposits confirmed fibrosis along with amyloidosis in the present case[5]. Further staining with Congo red and Masson's trichrome on tissue sections of other organs also revealed amyloid deposition with fibrosis.

Bacteriological culture of pus drained from plantar abscess produced round, smooth glistening white colonies of 1-4 mm size on nutrient agar (Fig. 4A). On Gram staining of smear from culture revealed group of cocci arranged in bunches indicating *Staphylococcus* species. Histopathologic examination of plantar swelling revealed mild diffuse fibrosis of the dermis around the necrotic area with heterophil infiltration and cocci bacteria (Fig. 4B). Gram-positive cocci were stained blue with the Brown and Brenn method of staining (Fig. 4C).

Although there are different biochemical types of amyloids exists in animals, the reactive type or AA amyloid type protein deposits predominate, which was made up of acute phase protein, serum amyloid A (SAA). Only systemic AA amyloidosis in domestic and caged wild birds has been documented[5]. Our observation of amyloid deposition in systemic organs other than the liver secondary to the chronic inflammatory bumble foot condition was in agreement with the observations of earlier reports[6,7]. The precursor protein SAA required for amyloid deposition in various tissues and organs were upregulated due to chronic inflammatory stimuli produced by pododermatitis condition on foot. Liver abnormalities, both gross and microscopic, were consistent with prior reports of severe hepatic amyloidosis in ducks[8,9]. Systemic amyloid deposition in liver and other organs such as ovary, kidney, spleen, pancreas and adrenals were reported in Japanese quail and Bengalese finch[10,11]. Avian amyloidosis is systemic type in nature and its development is associated with predisposing conditions such as age, breed, neoplastic conditions, stress conditions, chronic inflammation and infections[8]. A study on management of bumblefoot in duck demonstrated that *Staphylococcus* is one of the infectious

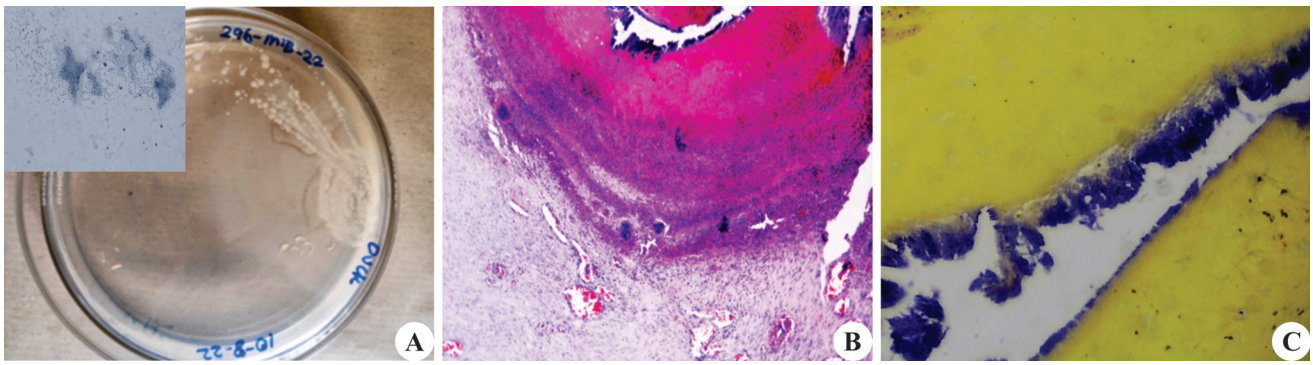

Fig. 4A. White smooth colonies on nutrient agar. Inset: Gram positive cocci on Gram's staining. **B.** Mild diffuse fibrosis of dermis with heterophil infiltration and bacteria in necrotic area (Foot, H&E x400). **C.** Blue colour cocci in dermal tissue (Foot, Brown and Brenn x400).

agents in the digital pad leading to chronic inflammation of the foot[11]. Since amyloid has an affinity to collagen[12], it is essential to assess the quantity of amyloid bound with collagen fibers to find the severity of damage caused by amyloid deposition in the connective tissue stroma of various organs. When stained with trichrome, amyloid that was attached to collagen fibers appeared red while amyloid without collagen fibre appeared blue[13]. Although the gold standard for amyloid detection remains Congo red staining, trichrome staining is an effective method to differentiate amyloid bound with or without collagen fibers[13]. Therefore, it is more appropriate to perform both trichrome and Congo red staining in amyloidosis to quantify the percentage of fibrosis in organs. Avian amyloidosis is a progressive fatal disease condition and amyloid fibrils deposited in tissues are relatively insoluble and resistant to physiological breakdown[14]. Due to the possibility of oral and interspecies amyloid transfer[15], it is required to conduct additional research on amyloidosis in birds used for food. Moreover, it is critical to distinguish between amyloid deposits, fibrosis, and their combination or extent in order to completely comprehend systemic amyloidosis in ducks.

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Title of Thesis	:	Pathomorphological studies on fipronil induced toxicity in male *wistar* albino rats and its amelioration with pomegranate peel extract *(Punica granatum)*
Name of the Student	:	**Dr P. Nakul**
Name of the Guide	:	Dr K. Sujatha
Degree/Year	:	MVSc/2024
Name of the University	:	Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh-517 502

Fipronil is a second-generation broad-spectrum phenyl pyrazole insecticide that is commonly used in veterinary, households, topical pet care products, and agricultural practices. The indiscriminate and inappropriate use of these insecticides causes various adverse effects in both humans and animals. Fipronil induces oxidative damage and impairs the functions of hepatic, nervous, nephrotic, reproductive, gastrointestinal, renal, hematologic, cardiovascular, and immune systems. To combat these complications, use of an herbal ameliorating agent with high antioxidant properties, fewer side effects, and better compatibility such as Pomegranate peel extract *(Punica granatum)* is one of them with enriched polyphenolic compounds.

The present study was carried out by procuring 24 male Wistar albino rats that were randomly assigned to four groups with six rats in each group. Fipronil was orally gavaged @ 10 mg/kg b. wt. using distilled water as a vehicle to group II animals. To study the ameliorative effects, *Punica granatum* @ 200 mg/kg b. wt. was fed along with fipronil to group IV for 6 weeks. Group I and III served as vehicle control and *Punica granatum* control respectively. In the present study, no mortality was observed in any of the treated groups. Significant ($P<0.05$) reduction in Hb, TEC, PCV, TLC, percent lymphocyte counts, and a significant increase in percent neutrophil count was recorded in fipronil treated group (II). These counts were significantly improved in *Punica granatum* ameliorated rats (group IV) when compared to fipronil treated group (group II).

Serum biochemical analysis revealed a significant ($P<0.05$) increase in AST, ALT, BUN, creatinine and a decrease in total protein, serum albumin, and globulin levels in group II animals than control animals. Significant ($P<0.05$) increase in lipid peroxidation values and decreased levels of SOD, catalase, GPx, and GSH levels in the liver, kidney, brain, and testis were observed in fipronil treated rats (group II) than Group I rats. All these biochemical and oxidative stress parameters were significantly improved in the *Punica granatum* ameliorated group rats (group IV). A Significant ($P<0.05$) decrease in serum T3, T4, testosterone, and elevated levels of serum TSH was observed in (group II) rats when compared to control rats. Fipronil treated rats showed a significant ($P<0.05$) decrease in epididymal sperm count and an increase in abnormal sperm count than control rats (Group I). Significant improvement in these values was observed in *Punica granatum* ameliorated rats.

Grossly, the lesions including paleness of the liver, atrophied spleen, cerebral blood vessel congestion, and congested kidneys were observed in fipronil treated rats (group II) when compared to control rats. There were no specific pathological changes in the ameliorated animals (Group IV).

Histopathologically, the liver of the rats of Group II revealed congested and dilated blood vessels, severe degenerative changes in hepatocytes, disruption of hepatic cords, dilated and congested sinusoids, periportal and portal infiltration of MNCs and fibroblasts, bile duct epithelial hyperplasia, focal loss of hepatocytes with infiltration of MNC's, fatty degenerative changes, necrotic changes with perivascular infiltration of MNC's. Whereas in the *Punica granatum* ameliorated rats (group IV), all these changes were very mild and the liver came to near normal in appearance.

Kidneys of fipronil treated rats showed severe degenerative and desquamated changes in renal tubules, perivascular edema, intertubular, and pockets of hemorrhages. Hypertrophied, degenerated, atrophied, and hyperplastic glomeruli, infiltration of MNC's in interstitial and periglomerular spaces. In *Punica granatum* ameliorated rats (group IV), these lesions were less severe. Brain sections of fipronil treated rats (Group II) included sub-meningeal hemorrhages, capillary proliferation, neuronal degenerative changes like shrinkage, central chromatolysis, satellitosis and neuronophagia, gliosis, spongiosis in the cerebral cortex, and rounding, shrinkage, and loss of Purkinje cells of the cerebellum. In *Punica granatum,* ameliorated rats (group IV) showed similar changes with reduced intensity. Testis of Group II rats showed thickened tunica albuginea, interstitial edema, variation in the size of seminiferous tubules, and widened lumen with separation of germinal cell layers, reduced number of Leydig cells in testis. Similar lesions were observed in *Punica granatum* ameliorated rats (group IV) with reduced intensity. Lungs of Group II revealed widened interstitial space with infiltration of MNC's and eosinophils, adipogenicity, degenerated and desquamated bronchial epithelium. In the *Punica granatum* ameliorated rats, these changes were very mild in intensity. Heart of fipronil treated group showed focal areas of sarcolytic changes, congested and thickened blood vessels, hemorrhages in between cardiac muscles, and mild infiltration of MNCs and fibroblasts. In *Punica granatum* ameliorated group, mild changes were noticed. Mild to moderate lymphocyte depletion was more cons-picuous in the spleen, lymph node, and thymus. In *Punica granatum* ameliorated group, very mild changes were noticed in these organs. Pancreas of fipronil treated rats revealed atrophied islets of Langerhans, hyperplasia of ductular epithelium, and replacement of acinar cells with fibrous tissue. The thyroid sections showed disrupted follicular structure with desquamation of lining epithelium and lack of colloids in the lumen of the follicles. The lesions in epididymis of Group II rats included reduced sperm density in the lumen, inter-tubular, and loss of stereocilia. The severity of these lesions was of lesser intensity in *Punica granatum* ameliorated rats (group IV) when compared to fipronil treated rats.

Immunohistochemically, fipronil treated rats showed increased expression of BAX marker in liver, testis and thyroid tissues whereas *Punica granatum* ameliorated rats showed less immunoreactivity with BAX antigen.

Title of Thesis	:	Pathomorphological and Immunohisto-chemical studies on uterus and ovary of domesticated queen cats
Name of the Student	:	**Dr G. Swetha**
Name of the Guide	:	Dr A. Nasreen
Degree/Year	:	MVSc/2024
Name of the University	:	Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh-517 502

The research on feline reproductive pathology is very nascent in India. Disorders affecting the ovaries and uterus can significantly impact the overall reproductive health and wellbeing of the animals. Cats, known for their independent nature and graceful demeanor, are often cherished as beloved companions, making their reproductive health a primary concern for owners and veterinarians alike. Understanding the complexities of reproductive tract abnormalities in these feline companions is crucial for timely diagnosis, effective management, and the preservation of their health and reproductive capabilities. Enhanced knowledge on basic reproductive pathology of the queen cat is essential for taking advantage of the techniques for assisted reproduction, diagnosis of uterine and ovarian pathologies in several species including wildlife. Hence, the present study was undertaken in order to establish the incidence of reproductive disorders and to determine gross, associated cytological, histopathological, immunohistochemical changes in various pathological conditions of feline ovary and uterus.

A total of 70 ovaries were collected from 35 queen cats irrespective of age and breed. Among them 51 ovaries (73%) showed different ovarian lesions such as nonneoplastic and neoplastic which accounted for 49% (34 cases) and 24% (17) cases respectively and the remaining 27% (19) ovaries were normal. The most common lesion noticed was follicular cyst in about 26 cases (37.14%) then followed by interstitial endocrine cell hyperplasia, the second most common lesion reported in thirteen ovarian sections (18.5%). The other lesions noted were luteal cyst in eight cases (11.4%), oophoritis in eight cases (11.4%), embedded corpusluteum in three cases (4.28%), follicular atresia in three cases (4.28%),cavernous hemangioma in three cases (4.28%), hemangiopericytoma in three cases (4.28%), histiocytoma in three cases (4.28%), hemangiosarcoma in two cases (2.85%), granulosa cell tumor in two cases (2.85%), polyoogonia in two cases (2.85%), folliculoid in two cases (2.85%), serous inclusion cyst in two cases (2.85) and smooth ovary inonecase (1.42%). Vascular disturbances were noticed in twenty cases (28.5) along with other ovarian lesions.

Gross observations revealed mild enlargement and irregular ovarian surfaces associated with different types of cysts. The most prevalent lesion, follicular cysts grossly exhibited as clear straw coloured fluid filled cyst with characteristic cytological and histopathological features. Interstitial endocrine cell hyperplasia was characterized by abnormal and excessive proliferation of interstitial endocrine cells around the cystic structures. Luteal cysts on gross examination showed thick and opaque walls. Oophoritis revealed focal to diffuse infiltration of inflammatory cells. Additionally, other functional disorders such as embedded corpus luteum, follicular atresia, serous inclusion cysts, folliculoids, polyoogonia, and smooth ovaries are noticed with unique histopathological features. Vascular disturbances were commonly noticed associated with these pathologies. Neoplastic conditions, such as cavernous hemangiomas showed mottled ovary which revealed large, dilated engorged blood vessels histopathologically. Hemangiopericytomas, histopathologically revealed characteristic fingerprint pattern of pericytes. Histiocytomas showed greyish white nodular structures grossly. Large, round cells with irregular borders with few mitotic figures were noticed cytologically and histopathological examination showed proliferation of neoplastic polyhedral histiocytes. Granulosa cell tumors showed radially arranged granulosa cells with centrally located Call Exner body on cytological and histopathological examination. Hemangiosarcomas were characterized by abnormal endothelial cell proliferation both in solid and capillary types.

Thirty five uterine samples were examined which showed 54.3% pathological lesions and other 45.7% were normal. Among 54.3 % the noninflammatory conditions were 31.4% and inflammatory conditions were 22.8%. Inflammatory conditions included acute and chronic endometritis in four cases (14.2%), cystic endometrial hyperplasia- pyometra (CEH-pyometra) complex in three cases (8.5%) and subacute endometritis in one case (2.8%). Noninflammatory conditions included, the non-neoplastic conditions such as uterine adenomyosis in eight cases (22.8%), endometrial glandular hyperplasia in four cases (11.4%), cystic endometrial hyperplasia in three cases (8.5%), mucometra in one case (2.8%), peri glandular fibrosis in two cases (5.7%), endometrial polyps in one case (2.8%), hemosiderosis in thirteen cases (37.4%) and neoplastic lesions included uterine leiomyosarcoma in one case (2.8%), uterine adenoma in two cases (5.7%), endometrial adenocarcinoma in one case (2.8%) and scirrhous adenocarcinoma in one case (2.8%).

Acute and Chronic endometritis exhibited glandular epithelial desquamation, mononuclear cell infiltration, and fibroplasia. CEH-pyometra complex was characterized by significant endometrial thickening with cystic structures filled with inflammatory debris. Non-inflammatory conditions included uterine adenomyosis characterized by dissemination of endometrial glands into myometrium, endometrial glandular hyperplasia with anormal proliferation of endometrial glands in mucosal layer, cystic endometrial hyperplasia characterized by hyperplasia of superficial glands of endometrium, mucometra showed bilateral distended uterine horns due to accumulation of mucoid fluid, periglandular fibrosis as adjoining lesion of chronic endometritis and endometrial adenocarcinoma. Polypoid projections of mucosal layer were noted in endometrial polyps. Neoplastic conditions such as uterine adenoma, uterine leiomyosarcoma, endometrial adenocarcinoma, and scirrhous adenocarcinoma showed distinct histopathological features. Hemosiderosis was observed in association with several uterine pathologies.

The immunohistochemical analysis identified key markers in uterine and ovarian pathologies of domesticated queen cats. Notably, strong cytokeratin expression was observed in epithelial cells of endometrial adenocarcinoma, scirrhous adenocarcinoma and cystic endometrial hyperplasia. Smooth muscle actin (SMA) was expressed in neoplastic muscle cells of uterine leiomyosarcoma. Vimentin showed strong

positivity in pericytes of ovarian hemangiopericytoma, while inhibin exhibited immunopositivity in granulosa and interstitial endocrine cells. Vascular endothelial growth factor (VEGF) was detected in stromal blood vessels, indicating its role in angiogenesis. These findings contribute to a better understanding of the molecular aspects of uterine and ovarian disorders in queen cats, enhancing diagnostic accuracy and treatment approaches in veterinary practice.

Finally, the study unveils the complexity of reproductive tract abnormalities in queen cats, emphasizing the prevalence of ovarian and uterine pathologies. It highlights the significance of thorough pathological examination of lesions such as follicular cysts, interstitial endocrine cell hyperplasia, endometritis, CEH-pyometra complex and few neoplastic conditions offering valuable insights and explaining the importance of early spaying in cats. The study also underscores the importance of immunohistochemical markers in characterizing these pathologies. This research was pivotal for enhancing diagnostic accuracy and treatment strategies, ultimately preserving the reproductive health of queen cats and advancing feline veterinary care.

Proceedings of Executive Committee (EC) / General Body (GB) Meeting of XXXX Annual Conference of the Indian Association of Veterinary Pathologists and XIV Annual Meeting of the Indian College of Veterinary Pathologists and National Symposium on "Advances in Veterinary Pathology for Diagnosis and Control of Emerging Diseases of Livestock and Poultry" held w.e.f. 20-22 December, 2023 at ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243 122, Uttar Pradesh, India

The Executive Committee / General Body Meetings were held under the Chairmanship of Dr B.N. Tripathi, President, IAVP on 20th and 21st December, 2023 at Bareilly, respectively. The meeting was attended by Vice Presidents, Secretary General, Joint Secretary, Treasurer, Web Manager, Managing Editor, Zonal Secretaries, EC Members and Life Members. Dr G.A. Balasubramaniam, Secretary General, IAVP welcomed all office bearers and EC/GB members. Dr B.N. Tripathi, President, IAVP gave opening remarks and appreciated the efforts of office bearers for advancement of IAVP/IVCP:

1. He insisted for the sincere efforts to be made to improve the NAAS rating of the Indian Journal of Veterinary Pathology and to include it in the Thomson Reuters Impact Factor and even seek professional assistance from recognized agencies.
2. Members should be encouraged to submit a large number of quality research papers to improve the standard of the IJVP.
3. He also emphasized that publication of one review article in each issue of the journal with an aim to enhance the citation of the journal should be continued.
4. The Veterinary Pathologists should be encouraged for appearing in the ICVP Diplomate examination and possibility of recognition of ICVP Diplomate by VCI may be explored.

Agenda No. 1: Approval of the minutes of the Proceedings of last Executive Committee / General Body meeting held at College of Veterinary and Animal Science, Rajendranagar, PVNRTVU, Hyderabad, Telangana

1. The Secretary General informed the members about the action taken report (ATR) based on the Proceedings of 39th IAVP Conference, 2022 held at PVNRTUV, Hyderabad, Telangana.
2. The Proceedings have been circulated among the EC members and all the members agreed with the same.
3. The approval of minutes of the 39th Proceedings of IAVP held at Hyderabad was proposed by Dr M. Saminathan, Joint Secretary and seconded by Dr Pawan Kumar, Treasurer. The minutes were approved by EC/GB members, respectively.

Agenda No. 2: Report by the Secretary General

1. **New Life Members:** A total of 39 new Veterinary Pathologists had been joined as Life Member of IAVP in 2023.
2. **Zonal Activities:** The Zonal Secretaries should strengthen their zonal activities of the Association by organizing the Webinars/Conferences/Workshops with more active participation of the members.
3. **Database Updating:** Updating of the addresses of Life Members of the IAVP was discussed and the Zonal Secretaries were requested to expedite the process and complete the work at the earliest.

Agenda No. 3: Report of the Chief Editor

Dr A. Anandkumar, Chief Editor, IJVP, could not turn up for the EC/GB meeting due to his personal reason and expressed his inability to attend the same in advance. On behalf of him, Managing Editor Dr Vidya Singh briefed the house, about the IJVP report and informed that:

1. During 2022-23, a total of 67 research articles and 5 thesis abstracts were received for publication in the IJVP.
2. All four issues of IJVP of 2023 were published well in time and uploaded in IAVP Website. The acceptance and rejection rate of the articles were 92.54% and 7.46%, respectively.
3 It was decided that the printing of invited review articles should not be charged from authors. It is prospective from 2024 onwards.
4. It was decided that IJVP should be linked with ICAR Online Review System. This responsibility was given to Dr R.V.S. Pawaiya and Dr Vidya Singh.
5. The Chief Editor and Associate Editor were requested to collect detailed reports of citations, downloads etc. of IJVP.

6. It was decided to conduct review meeting after a month to assess the progress of the journal.
7. The purchase of anti-plagiarism software is time being postponed.
8. The enhancement of editorial assistance salary was not approved.

Agenda No. 4: Non-return of the advance/seed amount (Rs 50,000/-) received by Dr M. Laxmanan, Organizing Secretary of XXXIX Annual Conference of IAVP during 17-20 November, 2022 held at Department of Veterinary Pathology, College of Veterinary and Animal Science, Rajendranagar, PVNRTVU, Hyderabad, Telangana

Matter was discussed in detail. It was decided that Dr M. Laxmanan, Organizing Secretary of International Veterinary Pathology Congress-2022 to return the seed money of Rs 50,000/- given for smooth conduct of the conference. He had collected the amount of Rs 47 lakhs. This amount was sufficient enough to conduct the conference in very comfortable manner. So, he should return the seed money at any cost. Further as per the IAVP regulation, 1/5th of the registration fee needs to be credited in the IAVP account.

Agenda No. 5: Approval of the newly formulated guidelines of IAVP-Prof. C. Balachandran Molecular Pathology Award

1. Newly formulated guidelines for IAVP-Prof. C. Balachandran Molecular Pathology Award were approved with minor modification (Point No. 5 & 6) and it should be included in the IAVP constitution along with Sessional Poster Awards.
2. It was decided that during the submission of abstract the authors need to specify the name of the award to which they are applying. For more information on award, the organisers are requested to furnish the URL address of IAVP website in the brochure itself.
3. For the convenience of the applicants, specific guidelines and proforma (checklist) need to be revised for all the four awards under Young Scientist Award category.

Agenda No. 6: Treasurer Report

Dr Pawan Kumar, Treasurer presented the Audit Report of the income and expenses of the IAVP for the year 2022-23. He informed the house:

Opening Balance for the year 2022-23	Rs 51,73,836.12
Income during the year	Rs 8,56,672.00
(Subscription fee, membership fee, publication charges, financial assistance from ICAR, interest earned from FDR, Award processing fee etc.)	
Expenditure during the year	Rs 8,04,028.16
(Printing IJVP charges, remunerations and salary, website maintenance, postal stamps, zonal conferences, audit fee, etc.)	
Loans/Advances (Hyderabad Conference and Zonal Conference)	Rs 54,130.00
Total Balance	**Rs 52,80,609.96**

Treasurer's Report was accepted by EC/GB members.

Agenda No. 7: Venue of the next IAVP conferences

1. The venue of the next IAVP Conferences was discussed in detail and Department of Veterinary Pathology, Faculty of Veterinary Sciences & Animal Husbandry, SKUAST-Jammu was finalized as the venue for the Veterinary Pathology Conference, 2024. Dr Pankaj Goswami, Professor & Head, SKUAST-Jammu was proposed as the Organizing Secretary of the Conference.
2. Department of Veterinary Pathology, Faculty of Veterinary Science & Animal Husbandry, Birsa Agricultural University, Ranchi, Jharkhand was proposed as the venue for the Veterinary Pathology Conference, 2025. Dr M.K. Gupta, Professor & Head was proposed to be the Organizing Secretary of the Conference.
3. Department of Veterinary Pathology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu was proposed as the venue for the Veterinary Pathology Conference, 2026. Dr. N. Pazhanivel, Professor was proposed to be the Organizing Secretary of the Conference.

Agenda No. 8: Condolence on Deceased IAVP Members

It was informed to the house that Dr M. Krishnan Nair, Retired Head, Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala; Dr V. Titus George, Retired Professor, Department of Veterinary Pathology, Madras Veterinary College, Chennai, Tamil Nadu and Dr Debi Prasanna Das, Assistant Professor, OUAT, Bhubaneswar, Odisha were passed away in the year 2023. The house appreciated the contributions and services of the individuals to the profession and IAVP and a silence of 2 minutes was observed for peace of the departed souls.

Agenda No. 9: Miscellaneous

1. Dr R. Somvanshi, Web Manager informed that IAVP website (https://www.iavp.org/) is regularly updated and functioning smoothly.
2. He also informed that The Lesion, 2023 was published and circulated to life members and also uploaded in IAVP website.
3. It was decided that all Zonal Secretaries should take a lead to conduct a Webinar/Workshop/Zonal Conference in first half of 2024. If not, Secretary General requested to send reminders to all the Zonal Secretaries.
4. General Secretary should write a letter to all the HODs of the Department of Veterinary Pathology to make all the PG students as life members of the IAVP.
5. It was decided that as per IAVP constitution regulations, the memento need to be given to the award winners in future.

Agenda No. 10: Vote of Thanks

Dr G.A. Balasubramaniam, Secretary General proposed the vote of thanks to all the members of the EC/GB for their contributions in fruitful discussion in the meeting.

NB: Proceedings prepared by Dr G.A. Balasubramaniam, Secretary General, IAVP and Dr M. Saminathan, Joint Secretary, IAVP and reviewed and edited by Dr R. Somvnashi, Web Manager.

RESULTS OF IAVP AWARDS - 2023

The Indian Association of Veterinary Pathologists has instituted a number of awards. Awards are distributed to Veterinary Pathologists who have excelled in professional competence as assessed by appropriate criteria. The IAVP administers more than 6 groups of awards where an application is required, the last date for receiving award application is one month before the opening day of conference. The awards results for 2023 are listed below:

I. IAVP-Young Scientist Awards

1. IAVP-Dr Balwant Singh Memorial Young Scientist Award for Best Oral Presentation

Title: Studies on elephant endotheliotropic herpesvirus hemorrhagic disease in Asian elephants with special reference to vascular endothelial dysfunction

Authors: Subash Athira, M. Karikalan, Kirtika Sharma, Gaurav K. Sharma, R. Saravanan, Arun Zachariah, R.V.S. Pawaiya and A.M. Pawde

Affiliation: Centre for Wildlife, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh

2. IAVP-Dr S.K. Nigam Memorial Young Scientist Award for Second Best Oral Presentation

Title: Etio-pathological characterization of retroviral infections in small ruminants and potential of multiple antigenic peptides for the development of diagnostics for SRLVs

Authors: Hiteshwar Singh Yadav, S.D. Neha, Vinay Kumar, Diwakar Singh Rana, C.P. Singh, Ajay Kumar, R.V.S. Pawaiya, Vidya Singh and Pawan Kumar

Affiliation: ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh

3. IAVP-Prof. S. Ramachandran Memorial Best Molecular Oncologist Presentation Award

Title: Immunohistochemical localization of cancer stem cells in canine mammary tumor using biomarkers

Authors: S. Sruthi, K.S. Prasanna, A.J. George, I.S. Sajitha, S.N. Sudeesh, P.P. Varuna and R. Bharathi

Affiliation: College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala

4. IAVP-Prof. C. Balachandran Molecular Pathology Award

Title: Exploring the molecular pathogenesis of endothelial injury induced by bluetongue virus infection in sheep

Authors: Deepti Singh, M. Saminathan, K.P. Singh, M. Dinesh, M. Philma Glora, Arun Chatla, D. Ranjith, R. Deva, S. Nandi, G.K. Sharma and S.K. Biswas

Affiliation: Centre for Animal Disease Research and Diagnosis, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh

II. (A) IAVP-Poster Presentation Awards

1. IAVP-Best Poster Presentation Award

Title: Hepatoprotective effect of *Inula racemosa* plant extract against DEN (Diethylnitrosamine) induced liver damage in murine model

Authors: Smriti Jamwal, Rakesh Kumar, R.K. Asrani, Ankita, Abhishek Verma, Joshi Gaurav Santosaro, Vikram Patial, Sonali Mishra and R.D. Patil

Affiliation: Dr G.C. Negi College of Veterinary and Animal Sciences, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh

2. IAVP-Organizing Secretary Second Best Poster Presentation Award

Title: Retrospective study on occurrence of neoplasms in Indian wild fields

Authors: Rahul G. Kadam, M. Karikalan, Arvind Mathur, A.M. Pawde and A.K. Sharma

Affiliation: Centre for Wildlife, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh

3. IAVP-Savithree Jibachch Sinha Third Best Poster Presentation Award

Title: Co-infection of porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus (PCV2) in pig population of Mizoram

Authors: Amitava Paul and Tridib Kumar Rajkhowa

Affiliation: CVSc & AH, Central Agricultural University, Selesih, Aizawl, Mizoram

II. (B) Sessional IAVP Poster Presentation Awards

Session: Molecular Pathology and Oncology

1. **Title:** Assessment of micro vessel density using CD 105 (endoglin) in correlation with vascular endothelial growth factor in canine mammary tumours
   Authors: V. Kumar, S. Ramesh, R. Thangathurai, M. Thangapandiyan, M. Parthiban, R. Ramprabhu and G.V. Sudhakar Rao
   Affiliation: Veterinary College and Research Institute, Tirunelveli, TANUVAS, Tamil Nadu
2. **Title:** Role of epithelial mesenchymal transition in the propagation of canine mammary tumor
   Authors: Priyanka, Nittin Dev Singh, Geeta Devi Leishangthem, Kuldip Gupta and Harmanjit Singh Banga
   Affiliation: College of Veterinary Science, GADVASU, Ludhiana, Punjab

Session: Pet/Companion Animals and Avian Pathology

1. **Title:** Study of pathogenicity of chicken anaemia virus in embryonated chicken egg
   Authors: Sedeneinuo Suohu, G.A. Balasubramaniam, A. Arulmozhi, T.R. Gopala Krishna Murthy and A. Raja
   Affiliation: Veterinary College and Research Institute, Namakkal, TANUVAS, Tamil Nadu
2. **Title:** Clinicopathological and molecular investigation of trypanosomiasis in a cat
   Authors: V.S. Degloorkar, V.A. Anandgaonkar, R.S. Ingole and B.P. Kamdi
   Affiliation: Post Graduate Institute of Veterinary and Animal Sciences, MAFSU, Akola, Maharashtra

Session: Laboratory, Wild Animals and Forensic Pathology

1. **Title:** Mucinous cholangiocarcinoma in captive sloth bears *(Melursus urcinus)*
   Authors: P. Sree Lakshmi, M. Karikalan, Subash Athira, Deekshita Vadapalli, S. Ilayaraja, Arun A. Sha, Pawan Kumar and A.M. Pawde
   Affiliation: ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh
2. **Title:** Effect of *Dalbergia sissoo* mediated silver nanoparticles against induced ulcerative colitis in mice
   Authors: T.C. Ashvini, R.S. Ingole, B.P. Kamdi, S.P. Waghmare, S.W. Hajare and A.K. Gade
   Affiliation: Post Graduate Institute of Veterinary and Animal Sciences, MAFSU, Akola, Maharashtra

Session: Farm Animals Pathology

1. **Title:** A rare occurrence of ocular and maxillary sinus histoplasmosis in a donkey
   Authors: V. Kumar, R. Thangathurai, N. Krishnaveni and M. Balagangatharathilagar
   Affiliation: Veterinary College and Research Institute, Tirunelveli, TANUVAS, Tamil Nadu
2. **Title:** Pathomorphological, bacteriological and molecular studies on *Mannheimia haemolytica* infection in ruminants
   Authors: Rakshita Sharma, Babu Lal Jangir, Gulshan Narang, Paras Saini, Ankit Magotra and Deepika Lather
   Affiliation: College of Veterinary Sciences, LUVAS, Hisar, Haryana

Session: Toxicopathology and Aquatic Animals Pathology

1. **Title:** Toxicopathology of dimethyl phthalate (DMP) in Wistar albino rats
   Authors: T. Prajwala, B. Kavitha Rani, S.S. Manjunatha, N. Prakash, S.J. Arun, K.N. Brunda and Akash Javoor
   Affiliation: Veterinary College, KVAFSU, Shivamogga, Karnataka
2. **Title:** Subacute oral toxicity of fenpyroximate in Wistar rats
   Authors: K.D. Fanase, V.A. Patel, D.M. Chaudhary, P.G. Solanki, G.D. Desai, D.P. Amin, V.S. Patel, S.H. Raval, R.S. Parmar, J.M. Patel and B.J. Patel
   Affiliation: CVSc & AH, Kamdhenu University, Sardarkrushinagar, Gujarat

III. Journal Awards, 2022

1. IAVP-Dr C.M. Singh Award for Best Full Research Article (Non-Pack Animals)

Title: *Lumpy skin disease: Pathomorphological features and molecular detection in dairy cattle of west coastal India*

Authors: Shivasharanappa Nayakvadi, Samruddhi Prasad Joshi, Susitha Raj Kumar, H.B. Chethan Kumar, Jagruti Bathini and Sanjay Kumar Uddarwar

Issue: *Indian J. Vet. Pathol.,* 46(2): 103-110, 2022; DOI: 10.5958/0973-70X.2022.00017.7

Affiliation: ICAR-National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, Karnataka

2. IAVP-Dr S. Damodaran Award for Best Oncology Paper/Case Report

Title: *Cross-talk between clinico-histopathological traits of malignant canine mammary tumors and their prognostic relevance*

Authors: Siddharth Gautam, Indrasen Chauhan, Chitra Joshi, Kuldip Gupta and N.K. Sood

Issue: *Indian J. Vet. Pathol.,* 46(1): 33-42, 2022; DOI: 10.5958/0973-970X.2022.00005.0

Affiliation: College of Veterinary Science, GADVASU, Ludhiana, Punjab

3. IAVP-Dr B.S. Rajya Award for Best Non-Oncology Short/Rapid Communication

Title: *Molecular diagnosis of infectious bursal disease outbreak in chickens in and around Aizawl district of Mizoram, India*

Authors: Jenifa Ahmed, Y. Damodar Singh, T.K. Rajkhowa, R.S. Arya, P. Roychoudhury and A. Kalita

Issue: *Indian J. Vet. Pathol.,* 46(4): 333-338, 2022; DOI: 10.5958/0973-70X.2022.00056.6

Affiliation: CVSc & AH, Central Agricultural University, Selesih, Aizawl, Mizoram

4. IAVP-Gang-Mana Sharma Award for Best Article/Case Report on Pack Animals

Title: *Diagnosis and immunohistochemical evaluation of equine preputial tumors*

Authors: M. Thangapandiyan, P. Krishnaveni, V. Kumar, P. Pothiappan and G.V. Sudhakar Rao

Issue: *Indian J. Vet. Pathol.,* 46(2): 150-152, 2022; DOI: 10.5958/0973-970X.2022.00024.4

Affiliation: Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu

IV. IAVP-Best Post Graduate Thesis Awards

1. IAVP-Best PhD Thesis Award

Thesis Title: Studies on chemically induced mammary tumor in Laboratory rat model and evaluation of anti-tumor effect of selected plant extract

Name of Student: Rakesh Kumar

Major Adviser: B.N. Tripathi, Vice-Chancellor, SKAUST, Jammu

2. IAVP-Dr Patri Rama Rao Memorial Second Best PhD Thesis Award

Thesis Title: Pathological and molecular studies on application of angiogenic biomarkers in canine epithelial tumors

Name of Student: V. Kumar

Major Adviser: S. Ramesh, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu

IAVP-Best MVSc Thesis Awards

1. IAVP-Prof. P.K.R. Iyer Memorial Best MVSc Thesis Award

Thesis Title: Studies on pathological conditions affecting Asian elephants with special reference to elephant endotheliotropic herpes virus hemorrhagic disease (EEHV-HD) in India

Name of Student: P. Sree Lakshmi

Major Adviser: M. Karikalan, ICAR-IVRI, Bareilly, UP

2. IAVP-Dr Ram Raksha-Kiran Shukla Award for Second Best MVSc Thesis

Thesis Title: Molecular classification of canine mammary tumors with special reference to triple negative phenotype: Correlation with prognostic markers

Name of Student: A.T. Faslu Rahman

Major Adviser: K.P. Singh, Joint Director, CADRAD, ICAR-IVRI, Bareilly, UP

V. IAVP-Achievement Awards in Specialty Subjects

1. IAVP-Best Farm Animals Pathologist Award

Monalisa Sahoo, ICAR-National Institute on FMD, International Centre for FMD, Bhubaneswar, Odisha

2. IAVP-Best Poultry Pathologist Award

Prashant M. Sonkusale, Nagpur Veterinary College, MAFSU, Nagpur, Maharashtra

3. IAVP-Dr B.L. Purohit Memorial Best Toxicologist - Pathologist Award

Madhav Nilakanth Mugale, CSIR-CDRI, Lucknow, Uttar Pradesh

4. IAVP-Best Wild life Pathologist Award

Bhavesh Trangadia, CVSc & AH, Kamdhenu University, Junagadh, Gujarat

VI. IAVP-Special Encouragement Awards

1. IAVP-Best Veterinary Pathology Teacher Award

D. Madhuri, CVSc, PVNRTVU, Rajendranagar, Hyderabad, Telangana

2. IAVP-Best Women Veterinary Pathologist Award

Rinku Sharma, ICAR-IVRI Regional Station, Palampur, Himachal Pradesh

VII. Fellowship of Indian Association of Veterinary Pathologists, 2023

Prof. T.K. Rajkhowa, Professor and Head, Department of Veterinary Pathology, College of Veterinary Sciences & Animal Husbandry, Selesih, Aizawl, Mizoram

VIII. IAVP Appreciations/Activities/Recognitions

1. IAVP-Dr P.P. Gupta Oration

Title: *Nervous system pathology: An overview*

Speaker: Dr Rajendra Singh

Affiliation: Former Head, Division of Pathology, ICAR-IVRI, Bareilly, UP

2. IAVP-Veterinary Pathology Congress - Thematic Lecture

Title: *Advances in veterinary pathology for diagnosis and control of emerging diseases in livestock and poultry*

Speaker: Dr C. Balachandran

Affiliation: Former Vice-Chancellor, TANUVAS, Chennai, Tamil Nadu

3. IAVP-Veterinary Pathology Congress - Continuing Veterinary Pathology Education Lecture

Title: *Novel approaches to rabies vaccines*

Speaker: Dr Jorge E. Osorio

Affiliation: Director, Global Health Institute, University of Wisconsin-Madison, USA

4. IAVP-Appreciation to Organizing Secretary and Team

Dr K.P. Singh, Organizing Secretary, Joint Director, CADRAD, ICAR-IVRI, Bareilly, UP

5. IAVP-President Appreciation Certificate for Best EC Worker/Chapter

Dr M. Saminathan, CADRAD, ICAR-Indian Veterinary Research Institute, Bareilly, UP

IX. IAVP One Time Special Memorial Lectures

1. Dr N.S. Parihar Memorial Lecture

Title: *Animal disease control in India: Chinks in the armor*

Speaker: Dr G. Saikumar

Affiliation: Principal Scientist, Division of Pathology, ICAR-IVRI, Izatnagar, UP

2. Dr M.K. Nair Memorial Lecture*

Title: Development of biomedical devices of animal origin-Cholederm: from mind to laboratory and then to clinics

Speaker: Dr T.V. Anilkumar

Affiliation: Scientist-G, Division of Experimental Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala

G.A. Balasubramaniam
(Secretary General, IAVP)