PATHOLOGY AND DIAGNOSIS OF *Theileria annulata* AND *Theileria orientalis* IN BUFFALOES

THESIS

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In partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

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BY

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2017

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I hereby declare that the experimental research work and Interpretation of the thesis entitled **"PATHOLOGY AND DIAGNOSIS OF *Theileria annulata* AND *Theileria orientalis* IN BUFFALOES"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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Date:

Place: Nagpur ***(Pratik N. Ingle Patil)***

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
AST	Aspartate transaminase
@	At the rate of
cumm	Cubic millimeter
mm3	cubic millimeter
DLC	Differential Leucocytic Count
ESR	Erythrocytic Sedimentation Rate
et al	et al/ibi) and elsewhere
etc.	et cetera) and the others
Fig.	Figure
g	Gram
gm/dl	gram per deciliter
H & E	Hematoxylin and Eosin
Hb	Hemoglobin
Hb %	Hemoglobin per cent
HP	Histopathology
HFCB	Holstein Friesian crossbred
hr.	Hour
KBB	Koch' blue bodies
MCHC	Mean corpuscular haemoglobin concentration
MCH	Mean corpuscular hemoglobin

MCV	Mean corpuscular volume
µl	micro liter
µg	Microgram
µg /L	microgram per liter
mcg/L	microgram per liter
mg	Milligram
mg/dl	milligram per deciliter
mg/kg	Milligram per kilogram
mg/kg b. wt	Milligram per kilogram body weight
mg/kg/day	milligram per kilogram per day
ml	Milliliter
viz	namely
NBF	Neutral buffered formalin
PCV	Pack cell Volume
PCR	Polymerase Chain Reaction
RBC	Red Blood Corpuscles
SEM	Scanning electron micrograph
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
i.e.	That is
TEC	Total Erythrocytic Count
TLC	Total Leukocyte Count

TSP	Total Serum Protein
TEM	Transmission electron micrograph
TNF	Tumor Necrosis factor
WBC	White Blood Corpuscles

INTRODUCTION

Animal health is one of the major contributing factors for national economy. Worldwide animal health is considered as key for improving national productivity. Animal wealth in India has increased manifold and animal husbandry practices have changed to great extent following the introduction of newer technologies like cross breeding and grading up of livestock.

Agriculture in India provides livelihood to about 70% of the population and employs about 65% of the work-force. Traditionally, livestock and dairy sectors have been treated as integral parts of agriculture; however these sectors have some unique characteristics of their own.

Livestock are important in supporting the livelihoods of poor livestock farmers, traders and labourers throughout the developing countries. Diseases affecting livestock can have a devastating effects on animal productivity and on trade of live animals, meat and other animal products, on human health and consequently, on the overall process of economic development.

Hemoprotozoan diseases cause devastating losses to the livestock sector and thus pose major constraints to the dairy industry throughout the World. These diseases play an important role amongst the agents responsible for undermining health of livestock. The distribution of vector borne hemoprotozoan diseases like Theileriosis, Babesiosis, Trypanosomiasis and Rickettsial diseases like Anaplasmosis, Ehrlichiosis are mainly governed by climatic conditions which vary with latitude, altitude and season.

Tropical theileriosis is an economically important disease of bovine, widespread in Southern Europe, North Africa, Middle East, Asia and India placing an estimated 200 million cattle population at risk (Purnell, 1978). The disease is caused by the protozoan parasite *Theileria annulata and T. orentalis,* which affects domestic cattle (*Bos taurus* and *Bos indicus*) and Asian buffalo (*Bubalus bubalis*). It has been reported that numerous species of tick vectors viz. *Boophilus, Dermacentor, Rhipicephalus, Hyalomma* and *Ornithodoros* can transmit *Theileria spp.* (Kahn and Line, 2006). Pathogenesis is primarily due to proliferation of infected leucocytes and anemia.

Bovine Tropical Theileriosis, an inapparent infection of indigenous cattle and buffaloes has emerged as one of the fatal disease of cattle and buffaloes since the early 1960s, due to large scale cross-breeding programme (Singh, 2002).

Buffalo population is mainly found in Asian countries like India, China, Japan, Pakistan, Nepal, Bangladesh, Philippines and Mediterian countries like Turkey and Italy as well as African countries like Kenya in Africa. India ranks first in buffalo population with approx. 108.7 million animals and is considered as one of the leading country in milk production. Hence, buffalo is a major milking animal as far as India is concern and also the draft animal in the state of Karnataka, Tamilnadu and Kerla.

Tropical Theileriosis threatens an estimated 200 million cattle and act as a major constrain for livestock production and improvement in many of the developing countries (Tait and Hall, 1990). It causes direct loss by mortality and indirect loss due to poor productivity, infertility and high cost of treatment. About 210 million dollars annual loss has been estimated in India (Singh, 1990). Devendra (1995) and Narladkar *et al*., (2000) estimated $ 800 million as annual economic loss due to Theileriosis in India.

Theilerial parasite are small dot like round to ovoid, irregular cocci or bacilii, rod, stick, comma, fusiform shaped, signet ring and pear shaped parasites with an apical complex composed of rhoptries in erythrocytes called as piroplasms. The schizonts seen in lymphocyte called as Koch's blue bodies. Theileria spp. Protozoan divide and multiply with in cytoplasm of lymphocyte.

The incubation period for Theileriosis ranges from 10 to 25 days. Considerable variation may be seen in clinical manifestations of the disease as per stage of infection. High degree of fever, anorexia, enlargement of superficial lymph node (parotid, prefemoral and prescapular), naso-ocular discharge, congestion of conjunctiva, hyper salivation, occasional diarrohoea and coughing are the symptom commonly observed in Theileriosis (Osman and Gaabary, 2007). During pyrexia, a lymph destructive phase associated with a pronounced leucopenia is seen (Brown, 1990). The disease is further characterized by a marked anemia by inducing erythrophagocytosis. Disease causes losses through rapid loss of body weight, milk production and mortality.

Field diagnosis of acute Theileriosis is based on clinical signs, vector distribution and detection of macroschizonts within leucocyte in Leishman or Giemsa-stained blood smear, lymph node and tissue impression smears. Confirmatory diagnosis is by the detection of schizonts in white blood cells or piroplasms in erythrocytes. This method is quick, easy and economical, however it is difficult to detect schizonts and piroplasms within blood cells in chronic and subclinical cases.

It is not always possible to obtain blood or lymph node biopsy smear suspected for Theileriosis at stage of high parasitemia for microscopic examination, which may lead to false negative results. In carrier stage of the disease, smear examination may not reveal piroplasms or Koch's blue bodies in erythrocyte and lymphocytes, respectively. Detection of piroplasms has low sensitivity due to less numbers of parasites in carrier animal leading to high false negative diagnosis of theileriosis in affected animals. (Mahmmod *et al.,* 2011).

In addition to traditional method of diagnosis by blood smear examination, molecular diagnosis provides specific, reliable and useful diagnostic tool for detect *Theileria* spp. and estimate the relative status of infection among herds; detect the chronic infection and is effective in evaluation on low parasitemia status of animal discriminating pathogenic vs non-pathogenic species of *Theileria* and other hemoprotozoans. Several workers have shown that PCR based method of detection for *Theileria* is more sensitive than conventional microscopic examination (Azizi *et al.,* 2008, Mahmood *et al.,* 2010). PCR can be useful for epidemiological studies for detection, control, prevention and determination of the immunological status of buffaloes.

Theileriosis is economically important disease in several Asian and African countries. There is paucity of information regarding prevalence of Theileriosis in dairy animals, (dairy cattle, buffaloes, sheep and goats) in India. Similarly, the reports on survey, diagnosis and control of Theileriosis in India in last decade is scanty (Kolte, 2017).

Keeping in the view, economic losses due to blood protozoan diseases especially Theileriosis faced by farming community, lack of sensitive diagnostic methods for detection of clinical cases and carrier animals and lack of cost

effective treatment for Theileriosis, the present work is designed with the following objectives:

1. Detection of Theileriosis in buffalo by PCR assay.
2. To compare sensitivity of blood samples collected from different anatomical locations *viz*., ear and jugular vein for detection of Theileriosis in buffalo.
3. To study haematobiochemical changes in blood of buffalo infected with Theileriosis
4. To study gross and histopathological lesions in buffalo succumbed with Theileriosis.

REVIEW OF LITERATURE

The review of literature has been categorized into following parts:

1. Blood smear examination and detection of Theileriosis by PCR assay.
2. Gross and histopathological lesions of *Theileria* spp. infection in buffaloes.
3. Haemato-biochemical alterations due to Theileriosis in buffaloes.

2.1 Blood smear examination and detection of Theileriosis by PCR assay

D'Oliveira *et al.,* (1997) screened total of 92 blood samples for *T. annulata* by blood smear examination, immunofluroscent antibody test and PCR assay and found positivity of 22 %, 40% and 75 % respectively. The author obtained that PCR was the most sensitive method detection of *Theileria annulata* even in low parasitemia state from blood samples from carrier cattle.

Roy *et al.,* (2000) carried out the amplification of small subunit ribosomal RNA gene of *T. annulata* spp. and detected *T. annulata* 84 % positivity by PCR and 16% by microscopic examination of blood smear.

Almeria *et al.,* (2001) reported PCR as the most sensitive and specific technique for detection of *T. annulata* and *Babesia* piroplasms in cattle from Minorca, Spain. The study concluded that PCR was found to be more efficient in detection of *Theileria* spp. In comparison to traditional diagnosis by blood smear microscopic examination.

Das and Ray (2003) conducted PCR assay on extracted salivary glands of *Hyalomma anatolicum* ticks from cattle of West Bengal, India and found 42.8% prevalence of *T. annulata* infection in salivary glands of ticks.

Jeong *et al.,* (2003) applied real-time PCR for diagnosis and quantification of *T. serengiti* by using specific primers of 33 kDa gene fragment. Comparison of TaqMan PCR with traditional microscopic examination of Giemsa's stained blood smear from cattle revealed specificity upto 0.00005% of parasitemia to traditional diagnosis. They further stated that molecular diagnostic method was not only applicable to detect chronic infection of *Theileria* but also effective in evaluation of parasitemia status of cattle, which could be used in monitoring the health status under field conditions.

Aktas *et al.,* (2006) diagnosed *Theileria* infection in cattle of Eastern Turkey by using species specific PCR and reported 41(16%) cases positive for piroplasms by microscopy and 114 (45%) cases positive for *Theileria* spp. by PCR. Out of 252 blood samples collected from cattle, total of 39% (99/252) of cattle were found positive for *Theileria annulata* and 7% (18/252) for benign *Theileria* spp (*T. serengiti / buffeli / oreintalis*) by PCR assay.

Azizi *et al*., (2008) studied 140 carrier state cattle for infection with Theleria annulata and reported 40% cases positive by pcr and 8.1% by Giemsa stained blood smears. The authors obtained higher sensitivity and accuracy of pcr based methods in comparison to blood smear examinations for the diagnosis of Theleria annulata infection in cattle

Mahmood *et al.,* (2010) evaluated sensitivity of blood smear examination and PCR assay for diagnosis of Tropical Bovine Theileriosis in 258 animals in Egypt. They found 18.6 % prevalence by microscopic examination of stained blood smear and 70 % by PCR. They further stated that specific PCR assays are more sensitive and accurate for the clinical diagnosis of Tropical Theileriosis.

Parthiban *et al.,* (2010) studied the incidence of *Theileria* infections in Tamil Nadu from suspected blood and tick samples using *Theileria* genus specific and *T. annulata* species specific primers by nested PCR assay. Out of 22 blood samples, only 3 samples showed the presence of *Theileria* parasite under oil immersion. The DNA extracted from blood and ticks were amplified by nested PCR for 1098-bp fragment using primers 989/990 (*Theileria* genus specific). N-PCR product was amplified using primers 989/1347 (*T. annulata* species specific) for 372bp fragment. They found 4 blood samples positive by nested PCR. Similarly out of 15 tick samples, only 2 samples were positive by nested PCR. They further stated that conventional PCR method works poorly because of sub optimal conditions like unpurified samples which can be salvaged, when compared with nested PCR assay.

Rady *et al.,* (2010) studied the incidence of *Theileria* in 150 Egyptian cattle and 35 buffalo by screening blood and lymph samples for PCR assay. They found the incidence of 65.6% and 45.3% in blood and lymph samples of cattle respectively, while in buffalo it was 16.7% and 25% in blood and lymph samples respectively. They stated that blood based PCR assay was the most sensitive for

detection of the *Theileria* infection in all cases (acute, chronic and carriers). Further they concluded that, *TAMS1* gene is most abundant and immunodomoninant antigen on the surface of merozoites and piroplasms of *Theileria annulata* can be used for target–based PCR which was most sensitive and specific for diagnosis of *Theileria* infection in either acute or chronic cases and also in carrier animals of Tropical Theileriosis.

Hoghooghi *et al.,* (2011) compared blood smear microscopy and PCR techniques for detection of *T. annulata* infection in carrier cattle of Golestan province of Iran. Total of 160 blood samples collected from apparently healthy cattle were examined for blood smear microscopy and semi-nested PCR assay for detection of *T. annulata*. Semi-nested PCR revealed 12 (7.5%) positive samples while Giemsa stained blood smear could detect 6 (3.75%) cases out of 160 blood samples. Sensitivity of Giemsa stained method was only 50%. They stated that semi-nested PCR technique is highly sensitive method and can be used for screening of *T. annulata* carrier cattle.

Kamau *et al.,* (2011) identified and characterized the species of *Theileria* in cattle belonging to six farms of New South Wales where disease outbreak occurred. Efforts were made for sub-typing of *T. orientalis* by type-specific PCR, sequencing of small subunit (SSU) rRNA gene and sequence analysis of the gene encoding a polymorphic merozoites / piroplasm surface protein (MPSP). Nucleotide sequencing of SSU rRNA and MPSP genes revealed the presence of four *Theileria* genotypes: *T. orientalis* (*buffeli*), *T. orientalis* (*ikeda*), *T. orientalis* (*chitose*) and *T. orientalis* type 4 (MPSP) or type C (SSU rRNA). The majority of animals showed mixed infections while few showed single infection. When MPSP nucleotide sequences were translated into amino acids, base transition did not change amino acid composition of the protein product, suggesting possible silent polymorphism.

Khukhuu *et al.,* (2011) conducted molecular epidemiological survey of *T. orientalis* in 94 yellow cattle, 43 water buffaloes, 21 sheep, 21 goats and 85 blood-sucking ticks of cattle in the Thua Thien Hue province of Vietnam. The major piroplasm surface protein (*MPSP*) gene of *T. orientalis* was detected by PCR from 13 cattle (13.8%), 11 water buffaloes (25.6%), 1 sheep (4.8%) and 9 ticks (10.6%). They conclude that phylogenetic analysis using *MPSP* gene sequences showed the presence of seven genotypes, four previously categorized

genotypes (Types 1, 3, 5 and 7) and three new genotypes (Types N-1, N-2 and N-3).

McFadden *et al.,* (2011) noticed haemolytic anaemia in cattle introduced from a presumed non-infected herd from south Otago to a herd in Northland, New Zealand, where Theileriosis is endemic. 11/29 (38%) of cattle showed anaemia showing haematocrit values <0.25 L/ L. *T. orientalis* (*T. buffeli/orientalis*) was confirmed by using PCR. The 18 S sequence data and phylogenetic analysis of *CoxIII* sequences showed samples had greatest similarity to *T. orientalis Chitose* from Japan. Previous reports have suggested that *T. orientalis* may be non-pathogenic in healthy cattle. However, this investigation provided evidence that pathogen is capable of causing clinical disease in cattle not necessarily debilitated by another disease.

Shahnawaz *et al.,* (2011) studied the prevalence of Tropical Theileriosis caused by *T. annulata* in cattle from southern Punjab (Pakistan). They compared PCR amplification and Giemsa-stained blood smear and found that, PCR amplification was more sensitive tool (19% parasite detection) as compared to blood smear screening (3% parasite detection) for the detection of *T. annulata* infection.

Yokoyama *et al.,* (2011) conducted molecular survey based on genes encoding the major piroplasm surface protein (MPSP) and p23 for *T. orientalis* detection in cattle grazing in southern areas of Japan, consisting of 2 farms in Kumamoto prefecture (Aso and Kuma districts) and 3 farms in Okinawa prefecture (Ishigaki, Iriomote, and Yonagunil islands). High prevalence rates of *T. orientalis* infection were seen in all cattle population by using diagnostic MPSP and p23 PCR assay. Phylogenetic analyses revealed 4 *MPSP* genotypes and 3 *p23* genotypes.

Kohli *et al.,* (2014) screened 301 blood samples from apparently healthy crossbred cattle from various locations of Dehradun district by using Giemsa's staining technique and PCR assay. Microscopic examination of blood smears revealed 27.2% (82) overall prevalence of Theileriosis. By genus specific PCR assay, 98 samples (32.5 %) were found positive for Theileriosis.

Chauhan *et al.,* (2015) collected a total of 52 blood samples from cattle suspected for Theileriosis across Banaskantha district of Gujarat. All samples

were screened for Theileriosis by Giemsa's staining technique and PCR. Total of 17 (32.69%) and 24 (46.15%) samples were found positive for Theileriosis by microscopic examination and PCR assay respectively. It revealed that microscopic technique has 70.83% sensitivity when compared with PCR technique. Thus, PCR technique may be recommended for screening of Theileriosis in area where high prevalence of diseases have been reported due to intensive dairy farming.

Khatoon *et al.,* (2015) collected blood sample from 155 cattle having typical clinical symptoms and screened by blood smear examination after staining with Giemsa stain and PCR for presence of *T. annulata.* PCR assay employs the primers specific for gene encoding 30-kDa major merozoite surface antigen of *T. annulata* and amplification of 721 bp was done. Out of total 155 animals, 34 were positive for *T. annulata* by blood smear method, whereas 134 samples were positive by PCR indicating more sensitivity of PCR when compared to conventional method of diagnosis.

Kundave *et al.,* (2015) collected total of 116 samples from infected as well as apparently healthy cattle and buffaloes for detection of *Theileria* by blood smear examination and PCR assay. Screening of blood smears by Giemsa staining detected 15 samples (12.93 %) positive for *Theileria* piroplasms. However, the PCR based screening using specific primers from major merozoite-piroplasm surface antigen sequence of *T. annulata* (*TAMS 1*) gene detected 74 samples (63.79 %) positive for *T. annulata* which also detected 59 samples that found negative by Giemsa staining and concluded that PCR based screening is more sensitive and accurate method for diagnosis of Theileriosis in cattle and buffaloes.

Sudan *et al.,* (2015) conducted study to evaluate duplex PCR assays for simultaneous detection of *Trypanosoma evansi* and *T. annulata* in buffaloes by using TBR F/ R and TAMS F/R as primer sets encoding for products of 164 and 721 bp for *T. evansi* and *T. annulata* respectively. The results were compared and correlated with conventional Giemsa-stained thin blood smear examination and single PCR assay. The duplex PCR detected each pathogen with the same level of sensitivity, irrespective of whether its DNA was amplified in isolation or together with DNA of another pathogen. Moreover, single and duplex PCRs were able to detect each species with equal sensitivity in serially diluted DNA representing mixtures of *T. evansi* and *T. annulata.*

Lawrence *et al.,* (2016) reported prevalence of epidemic of bovine anaemia associated with *T. orientalis* infection in New Zealand between 30 August, 2012 and 4 March, 2014. Blood samples collected were analyzed by using quantitative PCR (qPCR) assay for detection of *T. orientalis* (*Ikeda*). In the first 18 months, out of 496 case herds; 392 (79%) dairy and 104 (21%) beef herds were positive for *T. orientalis* infection. Of 882 individual cases, 820 (93.0%) were positive for *T. orientalis Ikeda* by qPCR assay.

Jinho *et al.,* (2017) investigated the epidemiology of *T. orientalis* infections in cattle of Republic of Korea (ROK) and characterized the genetic diversity of *T. orientalis* based on the major piroplasm surface protein (MPSP) genes. Blood samples were collected from 138 asymptomatic cattle belonging to different geographical regions (Hoengseong, Jeongeup, Namwon, and Jeju Island) of ROK. Total of 57 blood samples (41.3 %, 57/138) were found positive for *T. orientalis* based on PCR amplification of MPSP gene. A high prevalence of *T. orientalis* infection was observed in Jeju Island, while the infection rate was relatively low in Jeongeup. Phylogenetic analysis showed that isolates identified were belonged to four MPSP genotypes, specifically 1, 2, 3 and 7 type. The distribution of four genotypes varied considerably among the four regions. Types 1, 2 and 3 were detected in Jeju Island, whereas types 1 and 7 were found in Namwon, types 1 and 2 in Jeongeup and type 2 in Hoengseong. These results suggested that MPSP genotypes detected in study showed genetic diversity related to geographical location. Of the four MPSP genotypes, the prevalence of most pathogenic type 2 was relatively high in ROK.

Kolte *et al.,* (2017) collected total of 1052 bovine blood samples from apparently healthy animals from four different agro-climatic zones of Maharashtra state along with data on host type, sex and body score. Samples were screened by PCR for detection of five major tick-borne prevalence pathogens: *T*. *annulata*, *T*. *orientalis*, *B*. *bigemina*, *B*. *bovis and Anaplasma* spp. The results demonstrated that single and co-infection with tick-borne pathogens are common. Prevalence across the climatic zones was detected. Simplistic regression models predicted that host type, sex and location are all likely to impact on prevalence of tick-borne pathogens. In order to remove issues with autocorrelation between variables, a subset of the dataset was modeled to assess any impact of tick-borne pathogens infection on body score of crossbreed versus native breed cattle (breed type). There model showed significant association between infection with

tick-borne pathogens (particularly apicomplexan parasites) and poorer body condition for crossbreed animals. Their findings indicate potential cost of tick-borne pathogens carrier infection on crossbreed productivity.

2.2 Gross and histopathological lesions of *Theileria* spp. infection in buffaloes

Omer *et al*., (2003) reported most prominent lesions in number of young and adult Friesian cattle undergoing lethal *T. annulata* infection. Goss lesions includes jaundice, petechial and ecchymotic hemorrhages involving mucosal and serosal surfaces of many organs as well as body fat, splenomegaly along with hemorrhages, swollen and haemorrhagic lymph nodes, prominent haemorrhagic ulcers and petechae in abomasums, enlarged mesenteric lymph nodes and congestion, oedema and emphysema in lungs. Microscopically, lymph nodes revealed oedema, cortex consisting of dense lymphoid tissue and medulla contained very sparse lymphocytes. It also showed activated cells including macrophages and numerous large lymphocytes and macrophages revealed various forms of parasite. Splenic nodules were sparse, diminished in size and without germinal centers. White pulp showed several mononuclear cells with parasite forms and hyperplastic macrophages. Liver showed fatty change. Lungs were characterized by degeneration and lymphocytic infiltration. Abomasum showed desquamation of the surface epithelium, submucosal lymphocytic infiltration and degeneration and infiltration of tunica muscularis.

Sundaram *et al*., (2003) conducted post-mortem examination of a crossbred cow succumbed to the disease and revealed severe anaemia, pale mucous membrane, icterus, frothiness in tracheal passage, enlargement of spleen, distended gall bladder and catarrhal inflammation of intestine. Blood smears examination revealed presence of concurrent infection with *Anaplasma marginale* and *Theileria spp.*

Aparna *et al.,* (2011) observed splenomegaly, massive pulmonary edema, frothy exudates in trachea, ecchymotic epicardial and diffused endocardial hemorrhages, punched-out ulcers in abomasum and severe hemorrhagic duodenitis in adult crossbred bovines during necropsy. Microscopic examination of stained blood smear revealed *Theileria* in lymphocytes and RBC. Further, PCR of blood sample revealed *T. orientalis* specific $P^{32}/^{33}$ gene.

Microscopically, liver showed periportal sinusoidal dilatation, focal area of necrosis, moderate fatty changes, proliferation of lymphocytes over portal triad and apoptotic bodies in hepatocytes. Haemosiderosis and depletion of lymphocytes in white pulp areas were marked in spleen. Inflammatory exudates between inter-alveolar spaces, proliferation of septal cells and thickening of inter-alveolar septa were noticed in lung. Heart revealed extravasations of erythrocytes in endocardium, loss of cross-striations in the myocardium and infiltration of mononuclear cells. Moderate to severe loss of mucosal epithelium with desquamation, necrosis of mucosal and submucosal tissue were observed in abomasum. Small intestine showed necrosis of epithelial lining of villi, infiltration of mononuclear cells, engorged capillaries and extravasations of erythrocytes.

Panda *et al.,* (2011) observed emphysema, atelectasis, pneumonic changes, enlarged lymph node and punched out ulcers on abomasal mucosa during necropsy in cattle of costal area of Orissa died due to Theileriosis. Histopathologically, liver sections revealed fibrosis of the portal tract and perivascular area. Thrombosis, disruption of hepatic cords, dissociation of hepatocytes and Kupffer's cell proliferation was frequently observed. Kidney sections showed degenerative changes involving all parts of the kidney, distended Bowman's space with varying amount of fibrous tissues. Tubular epithelium both in cortical and medullary zones showed varying degree of degeneration and necrosis and distended with fibrinous exudates. Lungs revealed areas of emphysema, atelectasis, thickened alveolar wall due to congestion or cellular proliferation, pneumonic changes, oedema, congestion, fibrinous and haemorrhagic infiltration into the alveoli. Spleen and lymph node showed severe depletion of lymphocytes and sometimes atrophy. In some cases, abomasum showed focal necrosis and ulceration of the mucosa, cellular infiltration into lamina propria and marked oedema of the sub-mucosa and muscular layer.

Hussain *et al*., (2012) collected blood and tissue samples from 50 tick-infested cattle belonging to modern slaughterhouse of Sulaimaniyah province. Giemsa stained blood smear and PCR assay revealed positive for *T. annulata* infection. Gross pathological examination revealed lymph nodes enlargement, pulmonary congestion and edema and multiple pale areas in kidneys.

Microscopically, the principle pathological lesion was represented by a marked lymphoproliferative reaction within these organs.

Kakati *et al.,* (2015) noticed ulcers in abomasal mucosa, distended gall bladder, pulmonary oedema, tarry coloured sticky intestinal contents indicating hemorrhage from abomasal ulcers during necropsy of pregnant cattle from Northeast India having history of high fever, inappetance, nervous symptoms followed by recumbency and non-responsive to subsequent symptomatic treatment during illness. Microscopic examination of blood smear followed by PCR assay confirmed the presence of *T. orentalis* infection.

Acharya (2016) conducted necropsy of three dead animals which were diagnosed positive for Theileriosis. Post-mortem findings revealed enlarged lymph nodes, jaundice, swollen kidneys, punched out hemorrhagic ulcers in the abomasum. Histopathological examination of liver showed congestion, haemorrhage, necrosis in areas surrounding to central veins. Lungs revealed congestion, haemorrhage, infiltration into alveolar lumen. Kidneys showed increased glomerular activity with increased cellularity and decreased free space inside the Bowman's capsule. Degeneration, necrosis and sloughing of tubular epithelium were also evident. The spleen and lymph nodes revealed depletion of lymphoid follicles, congestion, haemorrhage, infiltration of macrophages and plasma cells. Abomasum showed ulceration with desquamation of the mucosal epithelial lining, congestion and infiltration of mononuclear cells in submucosa.

Gupta *et al*., (2016) observed gross lesions of enlargement of peripheral and visceral lymph nodes, punched out ulcers along with haemorrhages in abomasal mucosa, pale mucous membranes and petechial haemorrhages on various visceral organs during necropsy in the cattle died due *T. annulata* infection. Histopathologically, lymph nodes revealed lymphocytic proliferation along with infiltration of plasma cells and macrophages. In addition, section of lymph node showed presence of schizonts of *T. annulata* either extracellularly or intracellularly within many lymphocytes and macrophages. The mucosal epithelium of abomasum showed multifocal areas of erosion, necrosis and ulceration along with infiltration of lymphocytes, plasma cells and macrophages in lamina propria.

2.3 Haemato-biochemical alterations due Theileriosis

Sharma (1979) compared haematological parameters *viz.*, TEC, TLC, Hb, PCV and DLC of *T. annulata* infected carriers cattle (1-2% parasitemia) and uninfected control. Analysis of data obtained on various parameters revealed significant ($P<0.01$) difference only in TLC values of the two groups. Differences in PCV levels were also seen but they were not significant.

Yadav and Sharma (1986) studied alterations in biochemical parameters of experimentally induced *T. annulata* infection in cattle. They found decreased values of total protein and increased values of alkaline phosphatase and serum bilirubin after experimental infection.

Aulakh *et al.,* (1998) compared haematological parameters of seven *T. annulata* infected cross bred cattle with those of 5 non-infected cattle at farm in Ludhiana district of Punjab. The analysis revealed significant ($P<0.01$) reduction in Hb, PCV, TEC and TLC. Further, there was significant ($P<0.01$) elevation in percentage of lymphocytes and eosinophils, and significan**t** ($P<0.01$) decreased in percentage of neutrophils and monocytes.

Mehta *et al.,* (1998) found reduced Hb, TEC and PCV values in experimentally induced cases of Bovine Tropical Theileriosis.

Sandhu *et al.,* (1998) studied haemato-biochemical changes during experimental *T. annulata* infection in cross bred calves which revealed significant ($P<0.05$) progressive decrease in Hb, followed by leukopenia. Analysis of serum biochemical parameter revealed significant ($P<0.05$) increased in levels of ALT, AST, ALP, creatinine kinase, gamma-glutamyl transferase, uric acid, BUN, bilirubin. The concentration of total protein, albumin, glucose, cholesterol and calcium showed non-significant reduction when compared with healthy cross bred calves.

Singh *et al.,* (2001) studied biochemical changes in cross bred calves experimentally infected with *T. annulata* positive tick tissue stabilize through subcutaneous inoculation. The mean values of serum total protein, albumin and immunoglobulin concentration showed marked reduction when compared with pre-infection values.

Omer *et al.,* (2002) studied haematological profiles of 403 pure bred cattle naturally infected with *T. annulata* in Saudi Arabia. All *T. annulata* infected cattle had lower TEC, Hb, PCV, MCHC, PLT (total platelets) value and significantly ($P<0.05$) higher MCV values than the controls. They further noticed significant ($P<0.05$) decreased mean values of TLC and absolute lymphocyte, eosinophil and neutrophil counts in *T. annulata* infected cattle compared to the controls. The percentage of lymphocyte, eosinophil, neutrophil, monocyte and basophil in the affected cattle were not significantly differed from those of controls.

Omer *et al.,* (2003) studied the biochemical profiles in young and adult Friesian cattle infected with *T. annulata* in Saudi Arabia. They reported significant ($P<0.05$) decreased values of total protein, albumin, globulin, serum, creatinine, phosphorus, magnesium, potassium, iron and copper along with significant ($P<0.01$) increased in mean values of AST and bilirubin when compared with healthy cattle.

Muraleedharan *et al.,* (2005) studied the haemogram of cattle naturally infected with *T. annulata* and reported low TLC and Hb levels in 31.39% of cattle. The total leucocyte count showed leukocytosis (25.09%) or an inclination towards leukopenia (19.72%) and DLC showed lymphocytosis (44.94%) and neutrophilia (16.19%). Local cattle had marked leukocytosis and buffaloes showed leukocytosis with neutrophilia.

Aulakh and Singla (2006) studied clinical and biochemical alterations in Bovine Tropical Theileriosis. Haematological observations revealed normocytic hypochromic anemia, neutrophilia, lymphopenia and leukocytosis. Biochemical changes revealed significant ($P<0.01$) decrease in serum creatinine, total plasma proteins, albumin and globulin and significant ($P<0.05$) increased in blood urea nitrogen.

Col and Uslu (2006) studied haematobiochemical changes in Holstein Friesian cattle naturally infected with *T. annulata.* They recorded significant ($P<0.01$) reduction in mean values of TEC, PCV, Hb, TLC and thrombocyte count while mean corpuscular volume and reticulocytes was increased significantly ($P<0.01$). Fibrinogen concentration was slightly higher in infected group than that in control group. Further, significant ($P<0.01$) increased levels of serum AST,

ALT, bilirubin, creatinine and urea and significant (P<0.05) decreased concentration of glucose, total protein, albumin, triglycerides, cholesterol, calcium and phosphorus was noticed in *Theileria* infected cattle.

Ahmad *et al*., (2007) studied 46 clinical cases of lethal Theileriosis in buffaloes from Bahadurnagar (Okara) area which were diagnosed by microscopic examination of stained blood smears. Buffaloes showed high temperature (104.80± 1.2), enlarged parotid lymph node, inability to walk, anorexia, listlessness, moderate anemia and in a few cases corneal opacity. Significant (P<0.01) decreased values of Hb (8.28 ± 1.81 gm /dl), TEC (4.14±0.7 $x10^6$/ul), PCV(22.10 ± 0.8%) and TLC (4.95 ± 1.05$x10^3$/ml) was evident in *Theileria* affected buffaloes when compared to healthy buffaloes. The percentage of neutrophils was increased to 61.75 ± 2.77 and lymphocytes decreased drastically to 28.96 ± 1.45 as compared to healthy buffaloes. Bupravaquone (Butalex) found to be the choice of drug as the buffaloes recovered from the disease due to early diagnosis and treatment. Necropsy findings of dead buffaloes revealed excessive fluid in the peritoneal and pleural cavity and haemorrhagic ulceration of abomasum.

Durrani *et al.,* (2007) observed significant (P<0.01) decreased levels of PCV, TEC and Hb in buffaloes suffering from *T. annulata* infection for which they collected blood samples from 600 buffaloes suspected for *Theileria* and found total of 107 (17.8%) as positive cases in Jia Bagga District of Lahore.

Osman and Al-Gaabary (2007) carried out haematological studies on Tropical Theileriosis in water buffaloes (*Bubalus bubalis*) in Egypt and recorded significant (P<0.05) decreased values of Hb, TEC and TLC in infected buffaloes compared to healthy control.

Altug *et al.,* (2008) studied alterations in haemato-biochemical profile of cattle naturally infected with *T. annulata*. They recorded significant (P<0.05) decreased values of Hb, PCV and platelet count along with increased in mean of TLC. They also observed significantly (P<0.05) increased activity of serum AST, ALT and ALP.

Hasanpour *et al.,* (2008) investigated changes in blood values of 20 adult buffaloes (13 females and 7 males) suffering from severe Theileriosis and reported significant (P<0.05) decreased mean values of TEC, TLC and PCV in

infected animals. They also reported the low mean levels of sodium, calcium, phosphorus and potassium in infected animals than healthy animals along with significant (P<0.05) decreased level of serum potassium. Significantly (P<0.05) higher mean serum values of AST and ALT in infected animals was also noticed.

Saber *et al.,* (2008) studied haematological alterations in 100 crossbred cattle naturally infected with *T. annulata* in comparison with 50 healthy cows in Iran and found significantly (P<0.05) lower levels of serum total protein, calcium, cholesterol and triglycerides concentrations and increased levels of ALP, ALT, phosphorous, sodium, potassium, bilirubin and BUN than the healthy cattle.

Ananda *et al.,* (2009) observed reduced hemoglobin level to 8 gm/dl in *Theileria* affected animals. In severely infected cases, hemoglobin was reduced to 3 gm/dl, while TEC and PCV were decreased to 2.3 million/cu mm and 9% respectively. They further concluded that this might be due to damage caused by the organism inside the RBC's during their multiplication.

El-Deeb and Younis (2009) carried out clinical and biochemical estimation of blood and serum collected from *T. annulata* infected buffaloes and found significant (P<0.01) decreased TEC and Hb concentration in *Theileria* infected buffaloes compared to healthy ones.

Qayyum *et al.,* (2010) showed significant (P<0.01) decreased in the mean values of hemoglobin concentration, PCV, TEC, TLC in *Theileria* infected animals when compared to those of healthy animals.

Aparna *et al.,* (2011) also observed marked decreased in Hb, TEC, PCV values in cross bred cattle of Weaned district of Kerala which found positive for *T. orientalis* infection as confirmed by PCR assay. Further, they also noticed leucopenia with normal differential leucocytic response in affected cattle.

James *et al.,* (2011) diagnosed *T. orientalis* infection in cattle belonging to 10 herds of Northland region, which has not been previously found in New Zealand. Though the piroplasm was relatively benign, but can cause severe anaemia in heavily parasitized animals.

Khan *et al.,* (2011) reported significant (P<0.05) decreased values of TLC, PCV and Hb concentration in *Theileria* positive cross bred cattle when compared to non-infected controls. Significant (P<0.05) decreased levels of serum total protein, albumin, globulin, glucose, calcium, phosphorus, cholesterol

and triglyceride concentration was also evident. The mean values of serum bilirubin and ALT was significantly (P<0.05) increased in *Theileria i*nfected cattle compared with non-infected control group.

Mahmmod *et al.,* (2011) investigated natural *Theileria* infection in 44 buffaloes and cattle out of 50, which showed fever, enlargement of superficial lymph nodes, severe lacrimation, bilateral conjunctivitis, photophobia and corneal opacity. Haematological analysis revealed significant (P<0.05) decreased values of TEC, PCV, Hb and TLC in infected animals when compared to control group.

Acharya (2016) screened a total of 5237 clinically suspected cases of bovine tropical theileriosis by blood smear examination and reported 3876 positive for theilerial infection by blood smear examination. *Theileria* positive cattle revealed significant decreased mean values of Hb, PCV, TEC MCV, MCH and MCHC than apparently healthy animals. Leucopenia, lymphopenia and relative neutrophilia was also evident. Similarly, serum biochemical profiles showed significant decreased levels of serum total protein, albumin and increased levels of SGOT and SGPT in *Theileria* affected cattle. The amplification of *TAMS 1* gene by PCR revealed *T. annulata* infection in cattle.

Memon *et al.,* (2016) studied the prevalence of *T. annulata* in peri-urban and urban areas of Hyderabad, Pakistan around collected 2400 blood samples from buffaloes. Total of 1845 (76.87%) animals were found to be infested with ticks. The overall prevalence of ticks was 970 (80.8%) in peri-urban and 875 (72.91%) in urban regions of Hyderabad. Out of 1845 samples from tick infected animals, 1680 (91.05%) were found to be positive for *Theileria* species by Giemsa-staining method. The authors concluded that *T. annulata* infection in buffaloes produced adverse effect on erythrocyte and leukocyte indices, whereas platelet indices remained unaffected.

MATERIAL AND METHOD

Present study on "Pathology and diagnosis of *Theileria annulata* and *Theileria orientalis* in buffaloes" was carried out at Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur, India. During the study period, sample collection was carried out by collecting the blood and preparing blood smear from various anatomical locations (ear and jugular vein) of Theileriosis suspected buffaloes which showed drop in milk production, anorexia, inappetance, high temperature and not responding to antibiotic treatment. Total of 227 *Theileria* suspected buffaloes were screened during 2016-17. Blood smear were stained with Giemsa stain / Leishmn's stain and observed for *Theileria* parasite under light microscope. Molecular conformation of *Theileria annulata* and *Theileria orentalis* from suspected blood samples was carried out by DNA extraction followed by PCR assay.

3.1 Source of samples

The blood samples were collected from buffaloes suspected for theileria infection from in and around Nagpur region. The animals in production stage (milking) and some of the male buffaloes were also included in the study sample. Representative tissue samples were collected from buffaloes suspected to have died due to Theileriosis.

3.2 Chemicals

Chemicals of extra pure grade procured from Hi Media Laboratories Pvt. Ltd., Mumbai and molecular grade chemicals from Sigma were used during the present research work.

3.3 Glass wares

Borosil (India) glass ware were used after sterilization in hot air oven at 160 ^{0}C for one hour.

3.4 Collection of blood samples

Blood samples from buffaloes suffering from pyrexia of unknown origin and tick infestation were collected from various anatomical locations (ear and jugular vein) in EDTA coated 2 ml vial and plain vial. Samples were stored at 4°C

till further analysis. Blood smear was prepared from both anatomical locations for microscopic examination.

3.5 Examination of blood smear

Thin blood smear prepared from ear and jugular vein were stained with Giemsa / Leishmn's stain and examined for presence for Koch's blue bodies (microschizonts or macroschizonts) in lymphocytes / monocyte and piroplasms in erythrocytes and lymphoblasts, if any (Benjamin, 2001).

3.6 Haematological examination

A total of 227 blood samples collected in EDTA (2mg/ml) coated vial from *Theileria* suspected buffaloes were subjected to routine haematological analysis. Haematological parameters viz., hemoglobin (Hb), total erythrocyte count (TEC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total leucocyte count (TLC), differential leucocyte count (DLC) were estimated using autohaemoanalyzer (mindray model no. BC- 2800Vet)

3.7 Biochemical examination

Blood samples collected in 2 ml plain vaccutainers from *Theileria* suspected buffaloes were used for biochemical study. Serum was separated and stored at -20°C till further used. The serum samples were used for analysis of biochemical profiles to assess alterations, if any.

All biochemical estimations were carried out on semi biochemical auto analyzer (Model: Erba-chem -7, India) using biochemical kits manufactured by Coral clinical systems (Tulip group) and AGAPPE. Following biochemical parameters were studied.

i) Serum total protein

ii) Serum globulin

iii) Serum albumin

iv) Serum glutamic oxaloacetate transaminase (SGOT) / Aspartate amino acetate (AST)

v) Serum glutamic pyruvic transaminase (SGPT) / Alanine aminotransferase (ALT)

3.8 Gross and histopathological examination

Postmortem examination of buffaloes suspected to have died of Theileriosis were carried out to record the various gross lesions. Tissue sample from lung, liver, kidney, spleen, lymph node, abomasum and brain were collected in 10% buffered formal saline solution and were subjected to routine histopathological examinations as per the standard method (Luna,1968).

3.9 PCR detection of *Theileria* from blood samples

All the blood samples collected were subjected to DNA extraction and subsequently PCR assay was performed for confirmation by using *Theileria annulata* and *Theileria orientalis* specific primers for TAMS 1 and MPSP gene, respectively.

3.9.1 Isolation of DNA

The DNA from all the blood samples was isolated by Martin method (Martin *et al*., 2010) and was subjected to PCR assay for amplification of TAMS 1and MPSP gene.

Martin method for DNA isolation

1. The Blood samples were collected for DNA isolation by preparing saponin lysates as follows: For each sample, 2 microfuge tubes each filled with 400 µl of blood and 1 ml lysis buffer (0.22 % NaCl, 0.015 % saponin, 1 mM EDTA, pH 7.5).
2. The tube contents were mixed and then centrifuged at 10,000 rpm for 3 minutes and the supernatant was discarded.
3. The pellets in each tube were re-suspended in 0.75 ml lysis buffer and contents of the 2 tubes were combined.
4. The tubes were again centrifuged and supernatant was discarded. This process was repeated until the pellet was clear of haemoglobin.

5. The pellet was then re-suspended in 100 µl of 50 mM KCl, 10 mM tris-HCl pH 8.0, 0.5 % tween and 100 µg proteinase K per ml.
6. The tubes were incubated in water bath at 56^{o}C for 2 hours after which they were immediately stored at –20^{o}C. A 1 in 5 dilution of the lysate was made for use as PCR template.

3.9.2 Amplification of DNA by polymerase chain reaction (PCR)

Primers used in present study as both forward and reverse (Sigma) to amplify TAMS 1 gene sequences of *T. annulata* are listed as follows.

Table 1: Details of *T. annulata* specific primers for TAMS 1 gene

Gene	Primer sequences (5'-3')	Product size	Reference
TAMS1	F- TACTTGGTCGACCCATGTTGT CCAGGACCAC R-ATCTTGCTCGAGAAGGAAGTAAAGG ACTGATGA	846 bp	Khatoon *et al.,* (2015)

Table 2: Details of *T. orientalis* specific primers for MPSP gene

Gene	Primer sequences (5'-3')	Product size	Reference
MPSP	F-TACATCGTCGACCCATGTTGTCCA AGAGATCG TTCAAC R-ATCTTGCTCGAGGAGATAGTAGAATGCTG CGAGG	852 bp	Kolte (2017)

Table 3: PCR reaction component of *TAMS1* gene.

Component	Quantity (µl)
PCR master mix (2X)	12.5 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10 pmol)	1.0 µl
Template DNA	2.0 µl
Nuclease free water	8.5 µl
Taq polymarase (5 unit)	0.0µl
Total	25 µl

Table 4: PCR reaction component of *MPSP* gene.

Component	Quantity (µl)
PCR master mix (2X)	12.5 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10 pmol)	1.0 µl
Template DNA	2.0 µl
Nuclease free water	8.4 µl
Taq polymarase (5 unit)	0.1 µl
Total	25 µl

The cycling conditions in Master gradient cycler for ***TAMS 1* gene** were as follow:

Step I	Initial denaturation	95˚C for 5 min	1 cycle
Step II	Denaturation	95˚C for 30 sec	30 cycles
	Annealing	57˚C for 45 sec	
	Extension	72˚C for 1 min	
Step III	Final extension	72˚C for 05 min	1 cycle
Step IV	Hold	4˚C	

The cycling conditions in Master gradient cycler for ***MPSP* gene** were as follow:

Step I	Initial denaturation	95˚C for 7 Min	1 cycle
Step II	Denaturation	94˚C for 30 sec	30 cycles
	Annealing	51˚C for 45 sec	
	Extension	72˚C for 1 min	
Step III	Final extension	72˚C for 05 min	1 cycle
Step IV	Hold	4˚C	

3.9.3 Confirmation of PCR product

PCR product was loaded in agarose gel (1.0% agarose in 0.5X trisborate-EDTA buffer, ethidium bromide 0.5µg/ml) along with standard molecular size marker (100 bp DNA ladder). The gel was electrophoresed (Horizontal gel

electrophoresis system, Genaxy). Amplified products were separated on agarose gel and observed by ultraviolet transilluminator and photographed using a gel documentation system (Gel-Pro Analyzer, Syngene, USA).

Statistical analysis

The data generated was analyzed statistically using students T test as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Considering the economic loss due to blood protozoan diseases in animals especially Theileriosis encountered by farming community and lack of its sensitive diagnostic methods for detection of clinical cases and carrier state animals, the present study was carried on pathology and diagnosis of *T. annulata* and *T. orientalis* in buffaloes. During the study period, samples collection was carried out by collecting the blood and preparing blood smear from various anatomical locations (ear and jugular vein) of Theileriosis suspected buffaloes which showed drop in milk production, anorexia, in-appetence, high temperature and not responding to antibiotic treatment. Total of 227 buffaloes were screened thoroughly during 2016-17. Blood smears were stained with Giemsa stain / Leishmn's stain and observed for *Theileria* parasite under light microscope. Molecular conformation of *Theileria annulata* and *Theileria orentalis* from suspected blood samples was carried out by DNA extraction followed by PCR assay. Results of present study are summarized and discussed as given below.

1. Microscopic examination of blood smear prepared from ear and jugular vein of buffaloes.
2. Haematobiochemical observations of buffaloes as suspected with Theileriosis
3. Detection and confirmation of *T. annulata* (TAMS1 gene) and *T. orientalis* (MPSP gene) by PCR assay.
4. Gross and histopathological lesions in buffalo as suspected for Theileriosis.

4.1 Microscopic examination of blood smears:

Blood sample was collected from ear and jugular vein of 227 buffaloes suspected for Theileriosis. Blood smear was prepared and stained with Leishmn's or Giemsa stain for microscopic examination of Koch's blue bodies (microschizonts or macroschizonts) within the lymphocytes, monocytes as well as piroplasms within erythrocytes.

Microscopic examination of stained blood smear revealed comma, dot, round, ovoid, annular, ring, pear and irregular cocci shaped numerous *Theileria* in erythrocytes. In many cases, 20-30% of the erythrocytes showed the presence of piroplasms (Plate 1). In addition, anisocytosis, micrositosis and hypochromic erythrocytes were also observed in *Theileria* infected buffalo indicating mild to

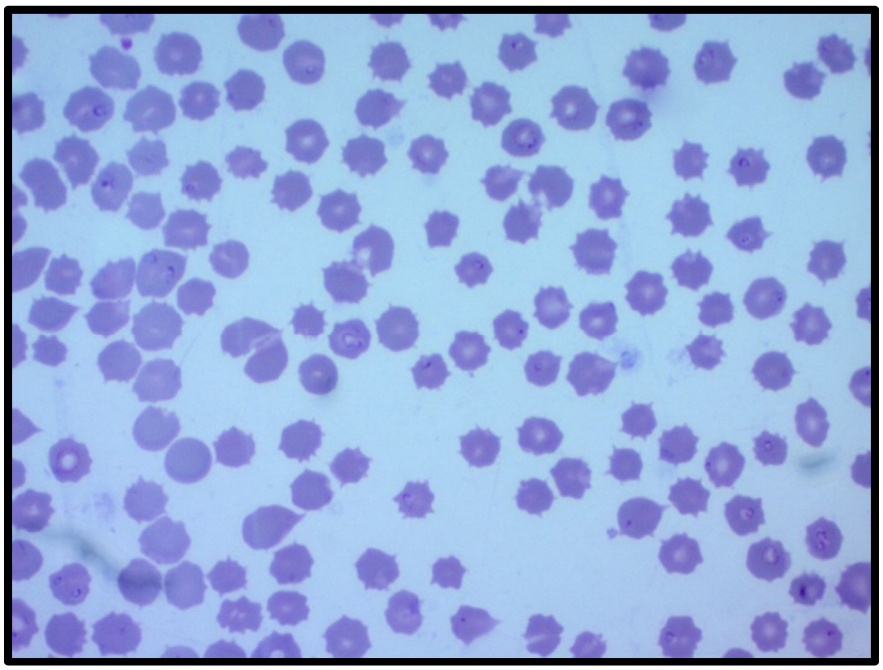

Plate 1: Erythrocyte showing various shapes of piroplasms. (Leishman's stain, 100X)

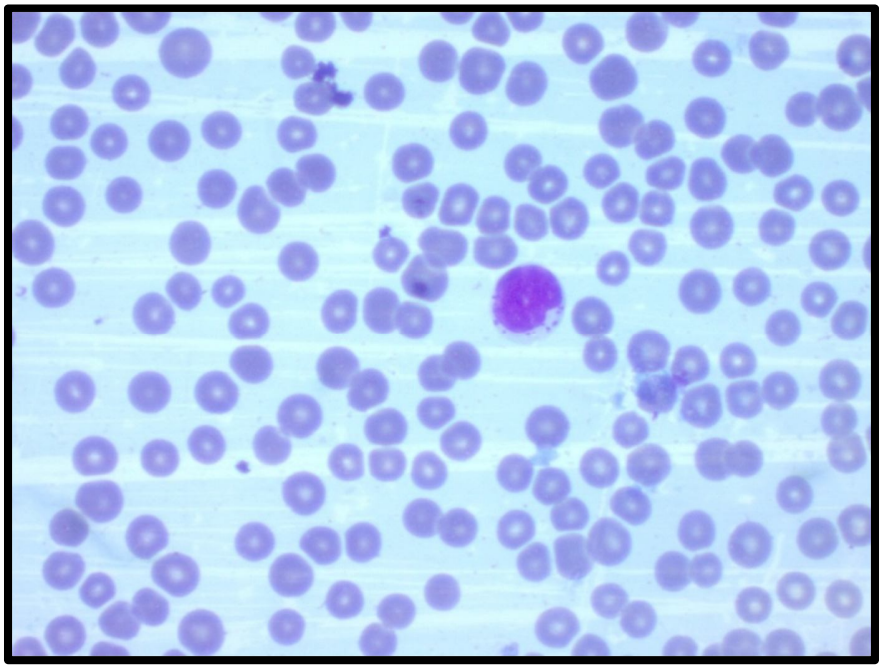

Plate 2: photograph showing lymphocyte infected with Koch's blue body and hypochromic RBCs. (Leishman's stain, 100X)

moderate anaemia (Plate 2). Various workers Gautam (1973), Dhar and Gautam (1979), Venugopal (1983), Bhatia *et al.,* (2001), Sastry and Rama Rao (2001), Mandal *et al.,* (2006), and have reported different forms of the piroplasms. Similarly, Sharma and Branco *et al.,* (2010), reported anisocytosis and polychromasia with the presence of immature erythrocytes in Theileria infected cattle.

Further, macro or microschizonts were also noticed in lymphocytes (Koch's blue bodies) and monocytes *Theileria* infected buffaloes (Plate 2, 3). Soulsby (1982) and Sastry and Rao (2001) also reported macro and microschizonts in lymphocytes of Theileria infected animals.

Total of 40 blood sample were found to be positive for *Theileria*, out of which 13 samples were from jugular vein and 27 samples were from ear vein. In this study, the prevalence of Theileriosis of 17.62% was recorded on the basis of microscopic examination of blood smear in and animal Nagpur region. The sensitivity of blood sample collected from jugular and ear vein was found to be 28.88% and 31.39 % respectively, while specificity for both was 100%. The present study inferred that ear vein can be the preferred site collection of blood samples in suspected cases of theileriosis in buffaloes.

Several workers have reported prevalence of Theileriosis by blood smears examination. 22% (D'Oliveira *et al.,* 1997), 16% (Roy *et al.,* 2000), 16% (Aktas *et al.,* 2002), 8.1% (Azizi *et al*., 2008), 18.6% (Mahmood *et al.,* 2010), *12.93%* (Kundave *et al.,* 2015), 32.63% *(*Chauhan *et al.,* 2015) reported prevalence of Theileriosis.

4.2 Detection and confirmation of *T. annulata* (TAMS1 gene) and *T. Orientalis* (MPSP gene) by PCR assay

For detection of *Theileria* spp. blood samples were collected form ear and jugular vein of 227 buffaloes suspected for Theileriosis. DNA was extracted from blood samples by standard methodology as described by Martin *et al*., (2010). The quality of extracted DNA was checked by gel electrophoresis and used for further procedure.

For the diagnosis of *T. annulata*, extracted DNA was further subjected to PCR for detection of TAMS1 gene by amplifying 846 bp gene fragment using

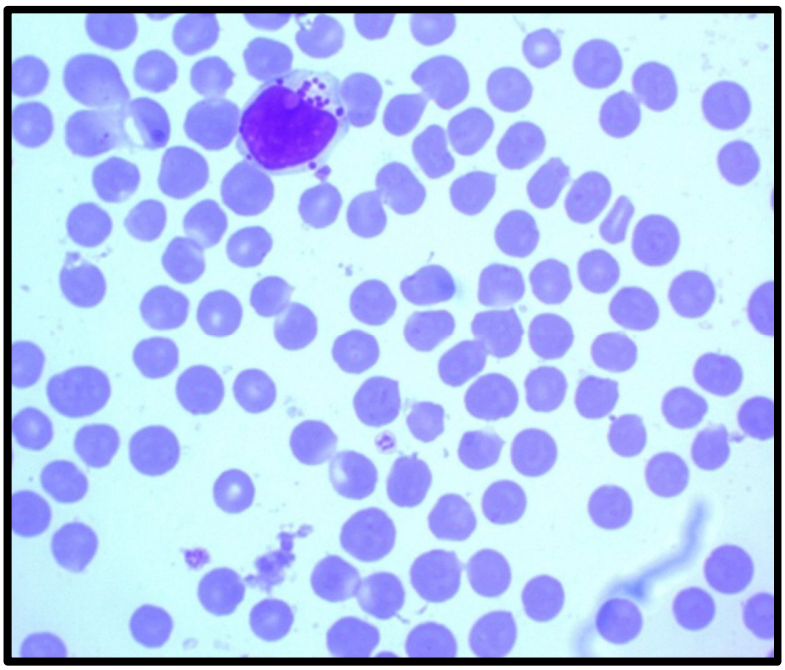

Plate 3: photograph showing lymphocyte infected with Koch's blue body.

(Leishman's stain, 100X)

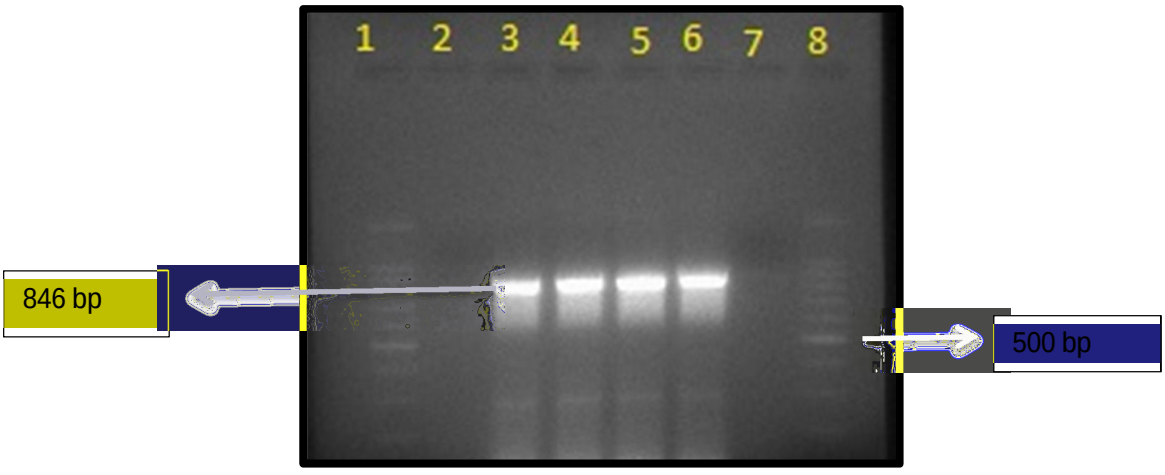

Plate 4: Agarose gel electrophoresis of PCR product of TAMS 1 gene.

Lane 1, 8 – Ladder (100 bp) **Lane 2, 7** - Negative Control

Lane 3 – Positive Control **Lane 4, 5, 6** – field sample

specific primers. Similarly, for *T. orientalis*, MPSP gene was detected by amplifying 852 bp gene fragment by using specific primers.

An expected amplified product of 846 bp of TAMS1 gene for confirmation of *T. annulata* was obtained from total of 69 blood samples (Plate 4). out of which, 33 blood sample were collected from jugular vein and 67 were from ear vein.

Similarly, for diagnosis of *T. orientalis*, an amplified product of 852 bp of MPSP gene was obtained from total of 13 blood samples (Plate 5), out of which, 8 samples were collected from jugular vein and 12 were from ear vein.

In addition, both TAMS 1 and MPSP gene was detected in total of 7 blood samples out of which 4 were from jugular vein and 7 from ear vein.

Several workers D'Oliveira *et al.,* (1997), Roy *et al.,* (2000), Almeria *et al.,* (2001), Aktas *et al.,* (2002), Jeong *et al.,* (2003), Das *et al.,* (2003), Jang et al., (2004), Aktas and Altay (2006), Azizi *et al.,* (2008), Parthiban *et al.*, (2010), Mahmood (2010), Rady *et al.,* (2010), Khukhuu *et al.,* (2011), Yokoyama *et al.,* (2011), Shahnawaz *et al.,* (2011), Hoghooghi *et al.,* (2011), Kamau *et al.,* (2011), Lan H. *et al.,* (2012), Kohli *et al.,* (2014), Kundave *et al.,* (2015), Sudan *et al.,* (2015), Chauhan *et al.,* (2015), Khatoon S. *et al.,* (2015), Jinho *et al.,* (2017) and Kolte *et al.,* (2017) earlier have evaluated usefulness of PCR in diagnosis of Theileriosis and reported that PCR was more sensitive technique in comparison to blood smear examination for the diagnosis of Theileriosis.

In the present study, out of total sample collected from 227 buffaloes, 27 and 13 samples collected from ear and jugular vein, respectively and were found to be positive by microscopic examination. suspected samples were subjected to PCR by using *Theileria annulata* and *Theileria orentalis* specific primers, 89 samples were found positive by PCR. Out of which 69 samples were positive for TAMS 1 gene (33 from jugular vein and 67 from ear vein) and 13 samples were positive for MPSP gene (8 from jugular vein and 12 from ear vein). In addition, a total of 7 samples were found to be positive for both, *Theileria annulata* and *Theileria orientalis* (4 from jugular and 7 from ear vein).

On the basis of PCR assay, prevalence of Theileriosis in buffaloes of Nagpur region was found to be 39.20 % (89/227). However, prevalence of *T. annulata*, *T. orientalis* and for infection with both (*T. annulata* and *T. orientalis)* in

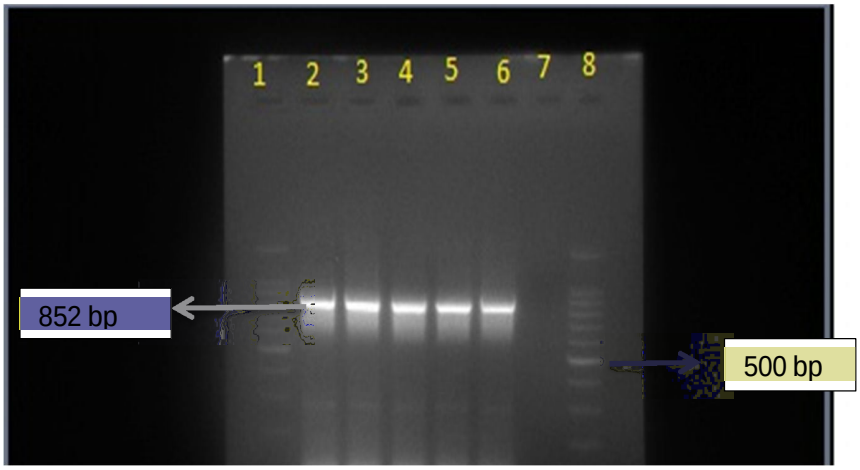

Plate 5: Agarose gel electrophoresis of PCR product of MPSP gene.

Lane 2 – Positive Control **Lane 3, 4, 5, 6** – field sample

Lane 7 - Negative Control **Lane 1, 8** – Ladder (100 bp)

Plate 6: photograph showing enlarge pre-sclapular lymph node.

buffaloes was found to be 30.39% (69/227), 5.72% (13/227) and 3.083% (7/227), respectively.

It was found that, the PCR technique was highly specific in the diagnosis of Theileriosis. Because of high pleomorphism among the piroplasms within the erythrocytes and ambiguity between KBB and azurophilic granules, the microscopic examination may likely provide false positive diagnosis in suspected cases of Theileriosis. Many times, in the chronic recurrent cases of Theileriosis treated by high doses of tetracycline for several days, the KBB or piroplasms are not visible by microscopic examination, giving false negative diagnosis of Theileriosis (D'Oliveira *et al*., 1997; Hoghooghi *et al.,* 2011).

4.3 Haematobiochemical observations of buffalo infected with Theileriosis

4.3.1 Haematological observations

A total of 227 blood samples from *Theileria* suspected buffaloes and 30 blood samples from apparently health buffaloes were collected and were subjected to estimation of haematological parameters viz; Hb, TEC, PCV, MCV, MCH, MCHC, TLC and DLC. Total Mean values of various haematological parameters of *T. annulata,* T. orentalis as well as both infections were compared with healthy buffalo.

Table 5: Mean values of haematological parameters in buffaloes

Parameters	Healthy Buffaloes	*T. annulata* positive buffaloes	*T. orentalis* positive buffaloes	Both *T. annulata* and *T. orientalis* positive buffaloes
Hb (g/dl)	11.54±0.46	9.37±0.25[a]	11.03±0.43	9.21±0.40 [β]
TEC (x10^6/ cumm)	7.16±0.27	6.30±0.16[a]	6.87±0.20	6.12 ±0.44 [β]
PCV (%)	39.74±1.67	31.61±1.15[a]	36.06±1.72	28.94±1.06[β]
MCV (fl)	55.88± 0.67	50.04±1.09[a]	53.05±1.79	47.40±1.64 [β]
MCH (pg)	16.21±0.14	13.56±0.24[a]	15.78±0.48	12.21±0.42 [β]
MCHC (%)	31.03±3.24	24.34±0.27[a]	28.91±0.82	22.82±0.89 [β]
TLC (x10^3/cumm)	10.99±0.19	9.66±0.25[a]	*10.19±0.26[A]	9.30±0.45 [β]

Lymphocyte %	62.60± 0.07	67.66±0.05[a]	64.69±0.19[A]	69.85±0.22[B]
Neutrophil %	32.30±0.08	27.42±0.04[a]	31.00±0.22	25.85±0.32[B]
Monocyte %	3.50±0.05	3.31±0.03	2.46±0.08	3.14±0.26
Eosinophil %	1.46±0.02	1.55±0.01	1.69±0.03	1.28±0.06
Basophil %	0.13±0.01	0.04±0.00	0.23±0.03	0.14±0.05

Mean with different superscripts in a row differ significantly (P< 0.01)

** Indicated mean with different superscripts in a row differ significantly (P< 0.05)*

4.3.1.1 Haemoglobin

The mean values of haemoglobin (Hb) of the healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in Table 5 and Figure1.

The mean values of Hb in healthy buffaloes, buffaloes found positive for *T. annulata*, *T. orientalis* and for both *Theileria* spp. were 11.54 ± 0.46, 9.37 ± 0.25, 11.03 ± 0.43 and 9.21 ± 0.40 g/dl respectively. The values of haemoglobin in *T. annulata* positive buffaloes was significantly (P<0.01) lower as compared to the healthy buffaloes. Similarly, haemoglobin values of the buffaloes found positive for both *T. annulata* and *T. orientalis* was also significantly (P<0.01) lower when compared to the healthy buffaloes. However, the value of haemoglobin in T. orientalis positive buffaloes did not differ significantly when compared with the healthy buffaloes, but was found to be non-significantly lower.

These findings are in a accordance with Aulakh *et al.,* (1998), Sandhu *et al.,* (1998), Muraleedharan *et al., (2005*), Col and Uslu (2006) and Altug *et al*., (2008) who observed significant (P<0.01) decreased values of Hb in cattle affected with *T. annulata*.

Similar findings were also reported by Durrani *et al.,* (2007), El-Deeb and Younis (2009) and Memon *et al.,* (2016) who reported significantly (P<0.01) decreased values of Hb in *T. annulata* affected buffaloes.

Significant decreased values of Hb were also reported by Aparna *et al*., (2011) and James *et al.,* (2011) in *T. orientalis* affected cattle. In our study, values of Hb in *T. orientalis* affected buffaloes were found non-significantly lowered when compared with healthy buffaloes.

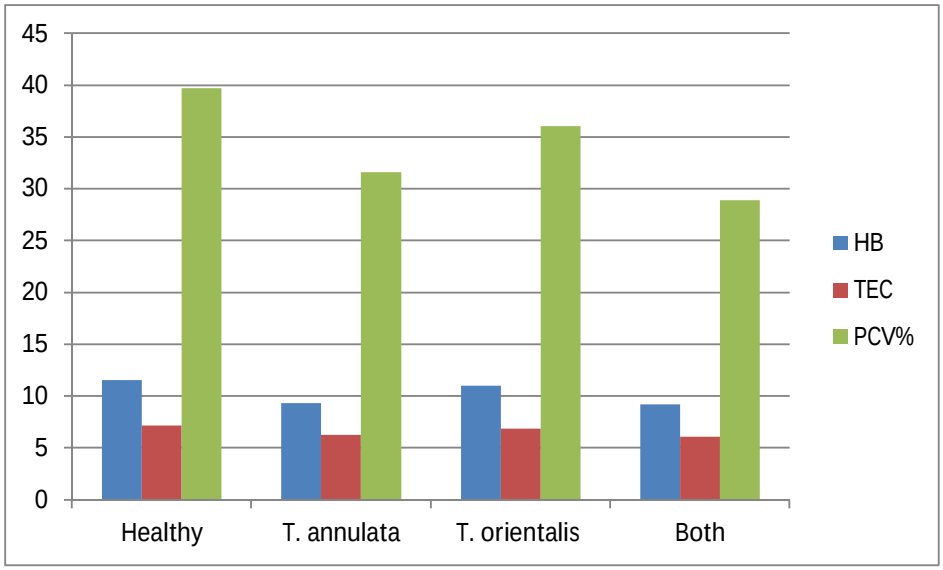

Figure 1:- Mean values of Hb, TEC and PCV in healthy and *Theileria* affected buffaloes.

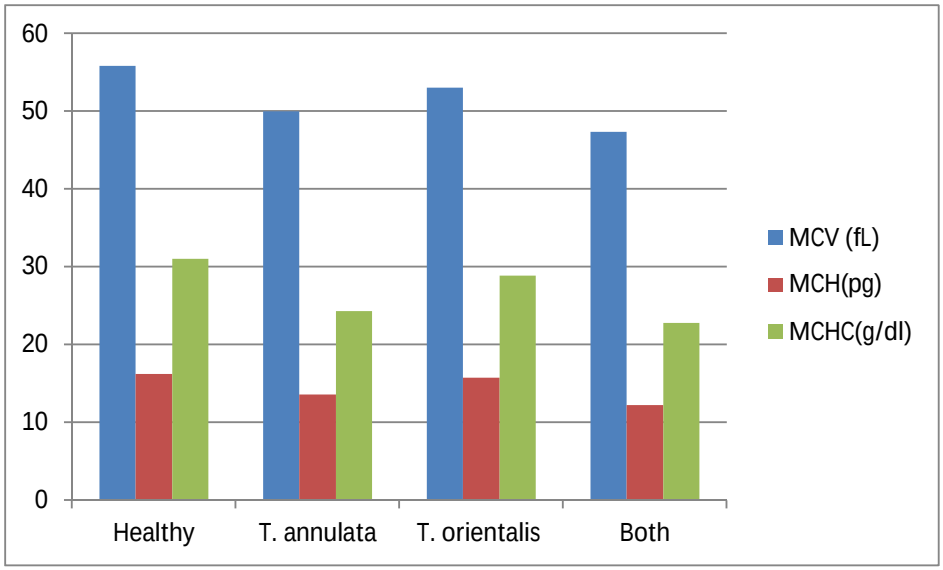

Figure 2:- Mean values of MCV, MCH and MCHC in healthy and *Theileria* affected buffaloes.

In addition, Ahmad *et al*., (2007), Osman and Al-Gaabary (2007) and Durrani *et al.,* (2007) also reported significant decreased values of Hb in buffaloes suffering from Theileriosis.

4.3.1.2 Total erythrocyte count

The mean values of TEC of healthy, T*. annulata, T. orientalis and for both T. annulata and T. orientalis* positive buffaloes are presented in Table 5 and Figure 1.

The mean values of TEC was 7.16 ± 0.27, 6.30 ± 0.16, 6.87 ± 0.20 and 6.12 ± 0.44 recorded in *T. annulata, T. orientalis and both T. annulata and T. orientalis* positive buffaloes, respectively. The data in the table indicate that *T. annulata* positive buffaloes showed significantly (P<0.01) decreased values of TEC when compared with the mean of healthy buffaloes. Similarly, significantly (P<0.01) decreased values of TEC was also noticed in the buffaloes positive for both *T. annulata* and *T. orientalis* infection as compared to healthy buffaloes. Whereas, mean of TEC in buffaloes which were positive for *T. orientalis* infection did not differ significant, but found lowered when compared with the mean of healthy buffaloes.

Aulakh *et al.,* (1998) and Col and Uslu (2006) also reported significant decreased values of TEC in cattle affected with *T. annulata*. While Omer *et al.,* (2002) observed lowered TEC in cattle affected with *T. annulata.*

Present findings are also in agreement with Durrani *et al.,* (2007), El-Deeb and Younis (2009) and Memon *et al.,* (2016) who reported significantly (P<0.01) decreased values of TEC in *T. annulata* affected buffaloes compared to healthy buffaloes.

Similarly, Aparna *et al*., (2011) and James *et al.,* (2011) also noticed significantly decreased TEC values in *T. orientalis* affected cattle. In present study, values of TEC in *T. orientalis* affected buffaloes were found non-significantly lowered when compared with healthy buffaloes.

Ahmad *et al*., (2007), Osman and Al-Gaabary (2007), Durrani *et al.,* (2007), Hasanpour *et al.,* (2008) and Mahmmod of *et al.,* (2011) also reported significant decreased values of TEC in buffaloes suffering from Theileriosis.

4.3.1.3 Packed cell volume

The mean values of PCV, of healthy *T. annulata, T. orientalis and for both T. annulata and T. orientalis* positive buffaloes are presented in Table 5 and Figure 1

The mean values of PCV in healthy buffaloes, *T. annulata* positive buffaloes, *T. orientalis* positive buffaloes and buffaloes found positive for both *T. annulata* and *T. orientalis* were recorded to be 39.74±1.67, 31.61±1.15, 36.06 ±1.72 and 28.94±1.06, respectively.

The mean values of PCV in buffaloes positive for *T. annulata* and for both *T. annulata* and *T. orientalis* was significantly ($P<0.01$) decreased compared to healthy buffaloes. While, non-significantly lowered values of PCV was observed in *T. orientalis* positive buffaloes.

Present findings are in agreement with Aulakh *et al.,* (1998), Col and Uslu (2006) and Altug *et al.,* (2008) who also reported significant decreased values of PCV in cattle affected with *T. annulata*. While Omer *et al.,* (2002) observed lowered PCV in cattle affected with *T. annulata.*

Durrani *et al.,* (2007) and Memon *et al.,* (2016) reported significantly decreased values of PCV in *T. annulata* affected buffaloes as compared to healthy buffaloes, which supports the findings of present study.

Similarly, Aparna *et al*., (2011) also noticed significantly decreased PCV values in *T. orientalis* affected cattle. In our study, values of PCV in *T. orientalis* affected buffaloes were found non-significantly lowered when compared with healthy buffaloes.

Ahmad *et al*., (2007), Hasanpour *et al.,* (2008) and Mahmmod *et al.,* (2011) also reported significant decreased values of PCV in buffaloes suffering from Theileriosis.

Decreased levels of Hb, TEC and PCV lead to anaemia which was more prominent in *T. annulata* and both *T. annulata* and *T. orientalis* positive buffaloes as compared to *T. orientalis* alone positive buffaloes. Study resumed that Anaemia results as the haemopoetic progenitors are suppressed by tumour necrosis factor-α (TNF- α) in Theileriosis leading to anaemic states of infected animlas. This could be attributed to the adverse effect on bone marrow by the toxic metabolites of *Theileria* spp. which interfere with the process of

erythropoiesis (Boulter and Hall, 2000). Further, anaemia may also results due to intra-erythrocytic piroplasms or auto-immune reaction resulting into destruction of infected erythrocytes as well as healthy erythrocytes (Hooshmand, 1976).

4.3.1.4 Mean corpuscular volume, Mean corpuscular haemoglobin and Mean corpuscular haemoglobin concentration

The mean values of MCV, MCH and MCHC of healthy, *T. annulata, T. orientalis and for both T. annulata and T. orientalis* positive buffaloes are presented in Table 5 and Figure 2

The mean values of MCV in healthy, *T. annulata, T. orientalis* as well as for both *T. annulata* and *T. orientalis* was 55.88±0.67, 50.04±1.09[a], 53.05±1.79, 47.40±1.64 respectively, while for MCH 16.21 ± 0.14, 13.56±0.24, 15.78±0.48, 12.21±0.42 respectively and whereas for MCHC, 31.03±3.24, 24.34± 0.27, 28.91±0.82, 22.82±0.89, respectively.

The buffaloes positive for *T. annulata* and for both (*T. annulata* and *T. orientalis)* showed significantly (P<0.01) decreased values of MCV, MCH and MCHC as compared to healthy buffaloes. Whereas, these values in *T. orientalis* positive buffaloes did not differ significantly, but were found to be lowered as compared to the healthy buffaloes.

These observations are in agreement with Omer *et al.,* (2002) and Col and Uslu (2006), who reported lower MCV, MCH and MCHC values during Theileriosis.

The mean corpuscular values are directly related to Hb, PCV and TEC. Therefore as Hb, PCV and TEC are affected in theileriosis, MCV, MCH and MCHC was also affected leading to microcytic hypochromic anemia in buffaloes.

4.3.1.5 Total leucocyte count

The mean values of TLC of healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in Table 5 and Figure 3

The mean values of TLC in healthy, *T. annulata, T. orientalis* and both *T. annulata* as well as *T. orientalis* positive buffaloes was 10.99 ± 0.19, 9.66 ± 0.25, 10.19 ± 0.26 and 9.30 ± 0.45, respectively. The buffaloes which were found positive for *T. annulata* and for both i.e. *T. annulata* and *T. orientalis* showed

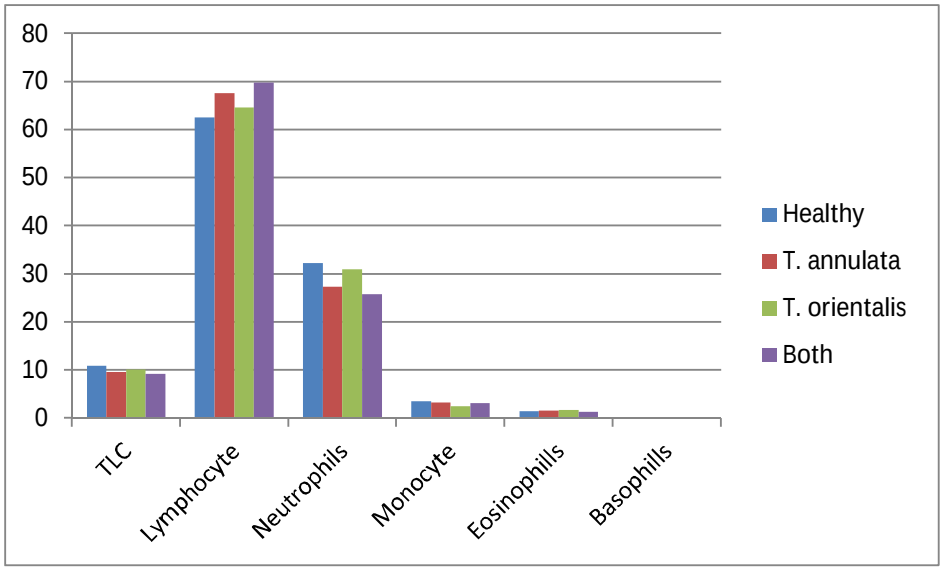

Figure 3:- Mean values of TLC, Lymphocyte, Neutrophils, Monocyte, Eosinophil and Basophils in healthy and *Theileria* affected buffaloes.

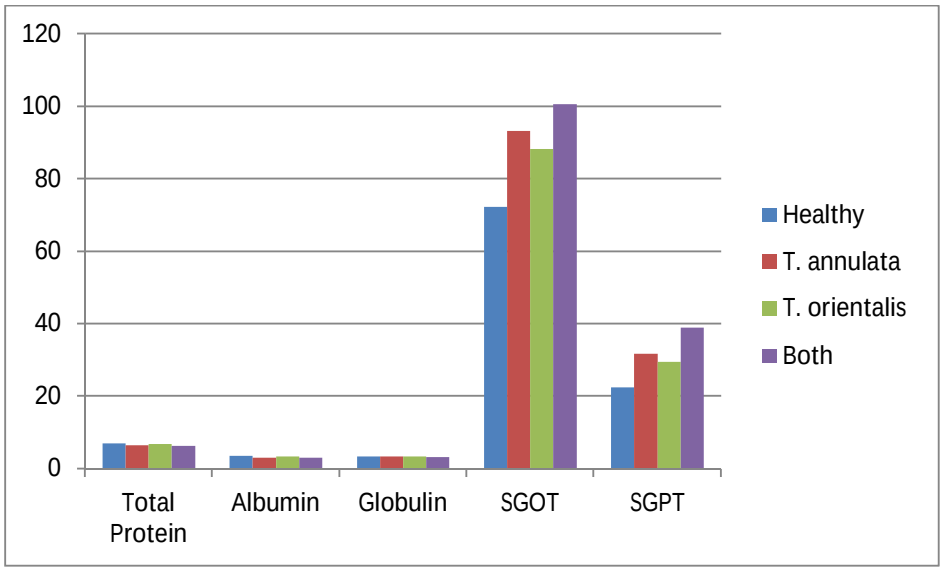

Figure 4:- Mean Value of Total Protein, Albumin, Globulin, SGOT, SGPT in healthy and Theileria affected buffaloes.

significantly (P<0.01) decreased values of TLC as compared to healthy buffaloes. Similarly, the values of TLC in *T. orientalis* positive buffaloes was also found significantly (P<0.05) lowered as compared to healthy buffaloes.

These findings are in accordance with Aulakh *et al.,* (1998), Omer *et al.,* (2002) and Col and Uslu (2006) who reported significant (P<0.01) decreased values of TLC in cattle affected with *T. annulata*. Similarly, Memon *et al.,* (2016) also reported significantly decreased values of TLC in *T. annulata* affected buffaloes. Further, Aparna *et al*., (2011) also observed significant decreased values of TLC in *T. orientalis* affected buffaloes.

In addition to this, Ahmad *et al*., (2007), Osman and Al-Gaabary (2007), Hasanpour *et al.*, (2008) and Mahmmod *et al.,* (2011) also reported significant decreased values of TLC in buffaloes suffering from Theileriosis.

Decrease values of TLC could be related to destruction of lymphocytes in lymphoid organs and infiltration of these cells into various organs (Clark *et al*., 1986). The histopathological finding of depletion of lymphocytes within spleen and lymph node in the present study supports leucopenia resulted to Theileria buffaloes

4.3.1.6 Differential leucocyte count (DLC)

The mean values of DLC of healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in table 5 and figure 3.

The mean values of lymphocytes in healthy buffaloes, buffaloes found positive for *T. annulata*, *T. orientalis* and for both *Theileria* spp. was 62.60± 0.07, 67.66±0.05, 64.69±0.19 and 69.85±0.22 respectively. The values of lymphocytes in *T. annulata* positive and both *T. annulata* and *T. orientalis* positive buffaloes was significantly (P<0.01) higher as compared to the healthy buffaloes. Whereas, *T. orientalis* positive buffaloes revealed non-significantly higher values of lymphocytes compared to healthy buffaloes.

The mean value of neutrophil of 32.30±0.08, 27.42±0.04, 31.00±0.22 and 25.85±0.32 was recorded in *T. annulata, T. orientalis and for both T. annulata and T. orientalis* positive buffaloes respectively. The neutrophil was significantly (P<0.01) decreased in *T. annulata* and both *T. annulata* and *T. orientalis* positive

buffaloes compared to healthy buffaloes. While buffaloes positive for *T. orientalis* showed non-significantly lower of neutrophils.

Mean of monocytes, eosinophil and basophil did not reveal any significant changes in the *Theleria* positive buffaloes when compared to healthy buffaloes.

These findings are in accordance with Muraleedharan *et al.,* (2005) and Acharya (2016) during *T. annulata* infection in cattle. Lymphocytosis could be due to early phase of infection, which may further leads to lymphocytopenia resultant to their destruction, which was evident in histopathology of lymph node and spleen.

4.3.2 Biochemical observations

A total of 69 blood samples were positive for *T. annulata*, 13 for *T. orientalis* and 7 for both *T. annulata* and *T. orientalis*. Mean values of various biochemical parameter of *T. annulata* positive buffaloes were compared with healthy buffaloes, *T. orientalis* positive buffaloes with healthy buffaloes and also the buffaloes found positive for both i.e. *T. annulata* and *T. orientalis* with healthy buffaloes by applying T- test for statistical analysis. Biochemical parameters like serum total protein, albumin, globulin, AST and ALT were estimated during the present study and the results are presented in Table 6.

Table: 6 Mean values of serum biochemical parameters in buffaloes affected with T*heileria*

Parameters	Healthy Buffaloes	*T. annulata* positive buffaloes	*T. orentalis* positive buffaloes	Both *T. annulata* and *T. orientalis* positive buffaloes
Total Protein (gm/dl)	6.94±0.07	6.52±0.07^{a}	6.77±0.15	6.36±0.18 $^{\beta}$
Albumin (gm/dl)	3.48±0.05	3.13±0.04^{a}	3.35±0.06	3.08±0.11 $^{\beta}$
Globulin (gm/dl)	3.46±0.06	3.38±0.05	3.41±0.10	3.28±0.08
SGOT/ AST (IU/L)	72.24±2.45	93.23±2.03^{a}	88.31±4.06^{A}	100.61±6.02 $^{\beta}$
SGPT/ ALT (IU/L)	22.54±1.01	31.74±0.99^{a}	29.48±1.90^{A}	38.96±5.32 $^{\beta}$

Mean with different superscripts in a row differ significantly (P< 0.01)

4.3.2.1 Serum total protein

The mean values of serum total protein of the healthy buffaloes and buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in Table 6 and Figure 4

The mean values of serum total protein in healthy, *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* positive buffaloes were found to be 6.94 ± 0.07, 6.52 ±0.07, 6.77 ± 0.15, 6.36 ±0.18 respectively.

Mean values of serum total protein in *T. annulata* and both *T. annulata* and *T. orientalis* positive buffaloes were found significantly (P<0.01) decreased as compared to the healthy buffaloes. However, mean values of serum total protein in buffaloes found positive for *T. orientalis* did not differ significantly, but were lower than the healthy buffaloes. Present findings of decreased value of serum total protein in *Theileria* infected buffaloes are in accordance with Yadav and Sharma (1986), Sandhu *et al.,* (1998), Omer *et al.,* (2003) Col and Uslu (2006), Saber *et al.,* (2008) and Acharya (2016), who have also reported significant decreased values of serum total in *T. annulata* positive cattle. Further, Aulakh and Singla (2006) also reported decreased levels of serum total protein in bovines during Theileriosis. Decrease in total serum protein values in liver the presence study in buffaloes naturally infected with Theileriosis could be due to decreased function possibly caused by hepatitis initiated by trapping of damaged infected erythrocytes and lymphocytes within the hepatic parenchyma.

4.3.2.2 Serum albumin

The mean values of serum total protein of healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in table 6 and figure 4

The mean values of serum albumin of 3.48±0.05, 3.13±0.04, 3.35±0.06 and 3.08±0.11 were observed in healthy buffaloes, *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* positive buffaloes, respectively. Values of serum albumin were significantly (P<0.01) lower in *T. annulata* and both *T. annulata* and *T. orientalis* positive buffaloes as compared to healthy buffaloes. The mean of serum albumin in *T. orientalis* positive buffaloes did not differ significantly, however values were found to be lowered compared to healthy buffaloes.

These findings are in agreement with Sandhu *et al.,* (1998), Singh *et al*., (2001), Omer *et al.,* (2003) Col and Uslu (2006) and Acharya (2016) where in significant decreased values of serum total in *T. annulata* positive cattle. Further, Aulakh and Singla (2006) also reported decreased levels of serum total protein in bovines during Theileriosis. Decreased values of serum albumin in buffaloes infected with Theileriosis could be due liver dysfunction possibly caused by inflammatory condition of liver initiated by trapping of damaged infected erythrocytes and lymphocytes interstitially in liver.

4.3.2.3 Serum globulin

The mean values of serum globulin of healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in table 6 and figure 4.

The mean values of serum globulin of 3.46±0.06, 3.38±0.05, 3.41±0.10, 3.28±0.08 were observed in healthy buffaloes, *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* positive buffaloes respectively.

In the present study, though the mean values of serum globulin did not differ significantly, but the mean of serum globulin found decreased in *T. annulata,* followed by both *T. annulata and T. orientalis* and only *T. orientalis* positive buffaloes compared to healthy buffaloes. The present findings of are in agreement with Omer *et al.,* (2003) and Aulakh and Singla (2006) who also reported decreased serum globulin in cattle due to Theileriosis.

Decrease in total serum globulin values could be due to lymphopenia resultant due to destruction of lymphocytes in lymphoid organs by the parasites. Further Histopathological lesions of spleen and lymph node also revealed depletion of lymphocytes, which also supports these findings.

4.3.2.4 Serum glutamate oxaloacetate transaminase (SGOT) / Aspartate transaminase (AST)

The mean values of SGOT of healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in Table 6 and Figure 4.

The mean values of SGOT in healthy, *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* positive buffaloes were found to be 72.24 ± 2.45, 93.23 ± 2.03, 88.31 ± 4.06 and 100.61 ± 6.02 respectively.

The mean values of SGOT of all buffaloes naturally infected with different *Theileria* spp. found significantly (P<0.01) higher as compared to healthy buffaloes. Significantly highest values of SGOT was observed in *T. annulata* positive buffaloes followed by both *T. orientalis* and *T. annulata* positive buffaloes and *T. orientalis* positive buffaloes when compared with the mean of healthy buffaloes.

The present findings of increased SGOT/AST levels in Theileriosis infected buffaloes were in accordance with those reported by Sandhu *et al.* (1998), Omer *et al.,* (2003), Altug *et al*., (2008) and Acharya (2016) in *T. annulata* positive cattle.

4.3.2.5 Serum glutamate pyruvate transaminase (SGPT) / Alanine transaminase (ALT)

The mean values of SGOT of healthy buffaloes and the buffaloes found positive for *T. annulata, T. orientalis and for both T. annulata and T. orientalis* are presented in table 6 and figure 4.

The mean values of SGOT in healthy, *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* positive buffaloes were found to be 22.54±1.01, 31.74±0.99, 29.48±1.90 and 38.96±5.32 respectively.

Significantly (P<).01) higher mean values of SGPT were noticed in all buffaloes affected with different *Theileria* spp. compared to the healthy buffaloes. *T. annulata* positive buffaloes showed significantly (P<0.01) highest level of SGPT followed by both *T. annulata* and *T. orientalis* positive buffaloes and only *T. orientalis* positive buffaloes.

These findings are in agreement with Sandhu *et al.,* (1998), Omer *et al.,* (2003) and Acharya (2016) who also reposted increased levels of SGPT in *T. annulata* positive cattle.

Increased levels of SGOT and SGPT in the buffaloes infected with *Theileria* spp. In the present study indicated liver damage either due to primary

or secondary hepatic necrosis and muscular dysfunction (Benjamin, 2001). The extensive erythrocyte damage caused by parasitic infestation (merozoite) result into severe anaemia leading to generalized hypoxia. This hypoxia could be one of the reasons for degenerative changes leading to necrosis. In addition, prolonged recumbency in Theileriosis also aid in muscle degeneration and necrosis.

4.4 Gross and histopathological lesions in buffalo died due to Theileriosis

During the study period, post mortem examination of six she buffaloes died due to *Theileria* spp. infection was conducted and pathomorphological changes were recorded. By PCR assay, it was found that three buffaloes died due to *T. annulata* infection, one buffaloes due to *T. orientalis* infection and two buffaloes due to both *T. annulata* and *T. orientalis infection.* The severity of lesions was more pronounced in buffaloes which were positive for both *Theileria* spp. followed by *T. annulata* and *T. orientalis.*

4.4.1 Gross lesions

Buffaloes which died due to *Theileria* showed frothy exudates in trachea, pulmonary congestion and emphysema (Plate 10). In some cases, oedema, consolidation and pneumonic changes were also observed. Pre-scapular lymph nodes were enlarged (Plate 6, 7). Congestion and enlargement of spleen along with rounded borders was also evident in all the cases (Plate 8). Catarrhal enteritis was seen in some buffaloes while in others mild haemorrhagic enteritis was noticed. Gall bladder was distended in few cases. In all the cases, the abomasum showed punched out haemorrhagic ulcers (Plate 9). Gelatinisation of peri renal fat and pale areas in kidney was observed. Some of carcass of buffaloes was anaemic and showed pale mucous membrane.

Similarly, Omer *et al*., (2002) also reported splenomegaly with haemorrhages, swollen and haemorrhagic lymph nodes, prominent haemorrhagic ulcers and petechae in abomasum, enlarged mesenteric lymph nodes and congestion, oedema and emphysema in lungs of Holstein Friesian cattle found to be positive *T. annulata* infection. Present findings are also in accordance with Panda *et al.,* (2011), who noticed emphysema, atelectasis, thickened alveolar wall, pneumonic changes, enlarged lymph node and punched out ulcers on abomasal mucosa during necropsy of cattle died to Theileriosis. The prominent gross lesions noticed in the present study are also in agreement with Aparna *et*

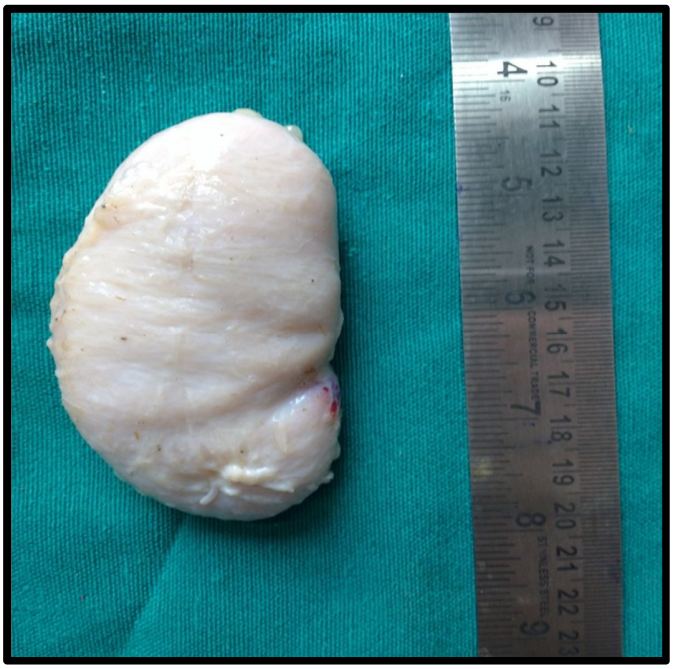

Plate 7: Buffalo died due to *Theileria* infection showed enlargement of pre-scapular lymph node.

Plate 8: photograph showing enlarged spleen.

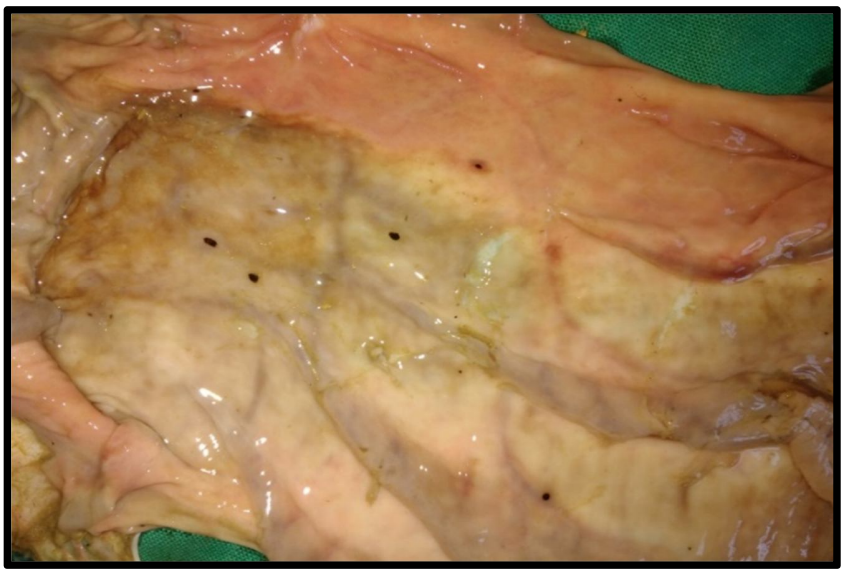

Plate 9: Buffalo from *Theileria* infection showing punched out ulcers

al., (2011) and Kakati *et al.,* (2015) who also noticed splenomegaly, massive pulmonary edema, and frothy exudates in trachea, punched-out ulcers in abomasum, severe haemorrhagic duodenitis and distended gall bladder during necropsy in cattle found positive for *T. orientalis* infection. Further, Acharya (2016) and Gupta *et al*., (2016) also reported gross lesions similar to our findings in cattle due Theileriosis.

4.4.2 Histopathological lesions

4.4.2.1 Spleen

Section of Spleen revealed marked depletion of lymphocytes from the germinal centers of white pulp of cortical region (Plate 11). Congestion and infiltration of macrophages was also evident. Lesions found in present study are in agreement with Omer *et al*., (2002), Aparna *et al.,* (2011), Panda *et al.,* (2011) and Acharya (2016) who also observed depletion of lymphocytes in spleen. Degeneration and Depletion of lymphocytes could be due to damaging effect of *Theileria* schizonts (Gill *et al.,* 1977).

4.4.2.2 Lymph node.

Prescapular lymph node from Theileriosis infected buffaloes revealed varying degree of focal and diffuse oedematous changes, congestion and focal lymphocytolysis. In most of the cases, section of lymph node showed medullary congestion, oedema and depletion of lymphocytes. (Plate 12, 13, 14). These findings are in accordance with Panda *et al.,* (2011) and Acharya (2016), who observed congestion, haemorrhages and depletion of lymphocytes in lymph node of cattle due to Theileriosis. Similarly, Omer *et al*., (2002) reported oedema, medulla showing very sparse lymphocytes in section of lymph node of cattle died due to *T. annulata* infection. While Hussain *et al*., (2012) and Gupta *et al.,* (2016) noticed lymphocytic proliferation in lymph node of cattle died due to *T. annulata* infection.

Enlargement and lymphproliferative changes could be attributed to lymphoproliferative nature of the disease induced by these parasites, the outcome of which had been represented by enlargement of the draining lymph nodes particularly in its early phases (Tait and Hall, 1990). And another possible explanation for this fact could be the cells infected by *Theileria* schizonts induce non-specific T lymphocyte proliferation (Cambell *et al.,* 1995) resulting in

Plate 10: froth in trachea, emphysema and congestion in lung of Buffalo died due to *Theileria* infection.

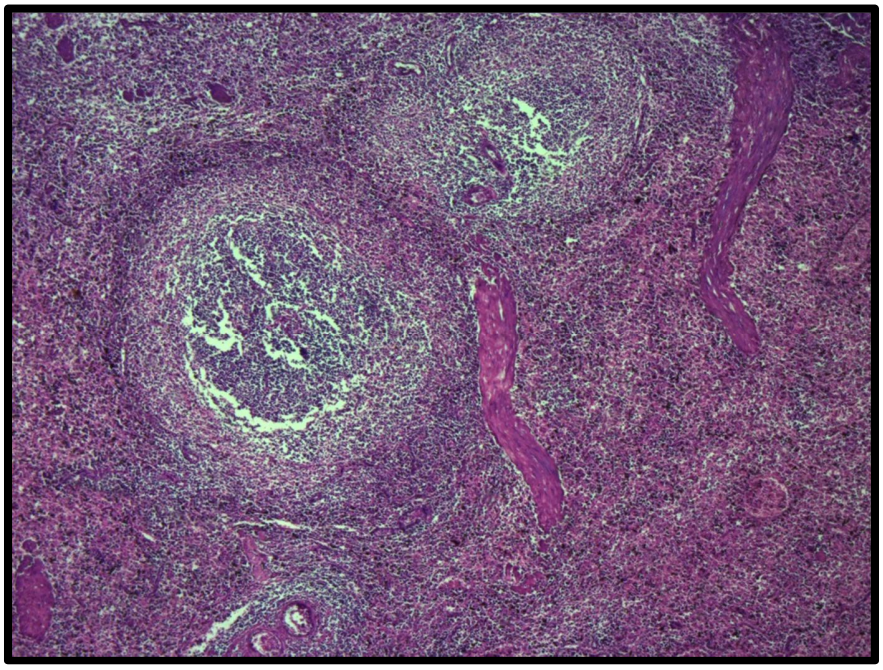

Plate 11: Section of spleen showing depletion of lymphocytes in white pulp of cortex. (H&E 20X)

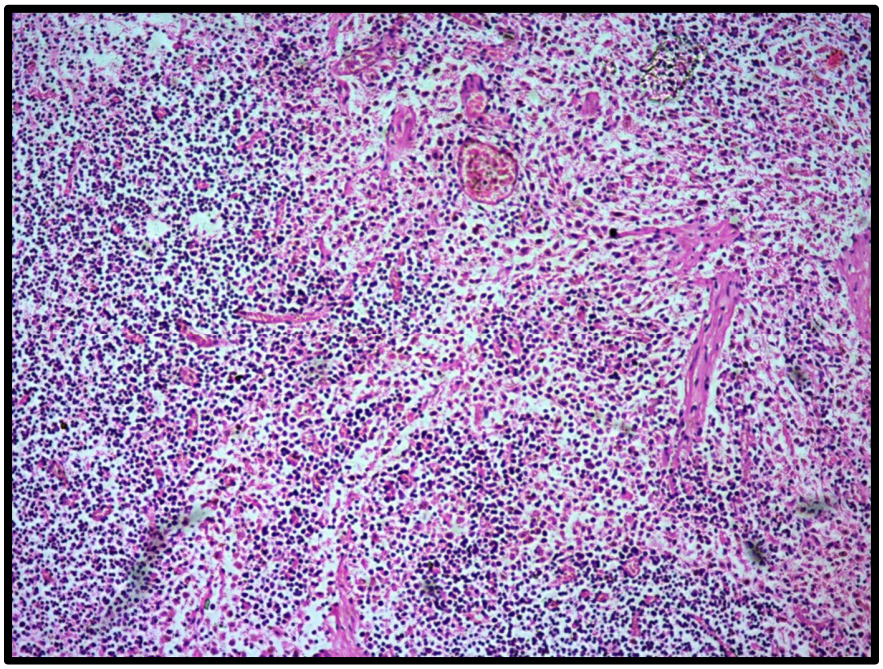

Plate 12: Section of lymph node showing congestion and mild depletion of lymphocytes in medulla. (H&E 50X)

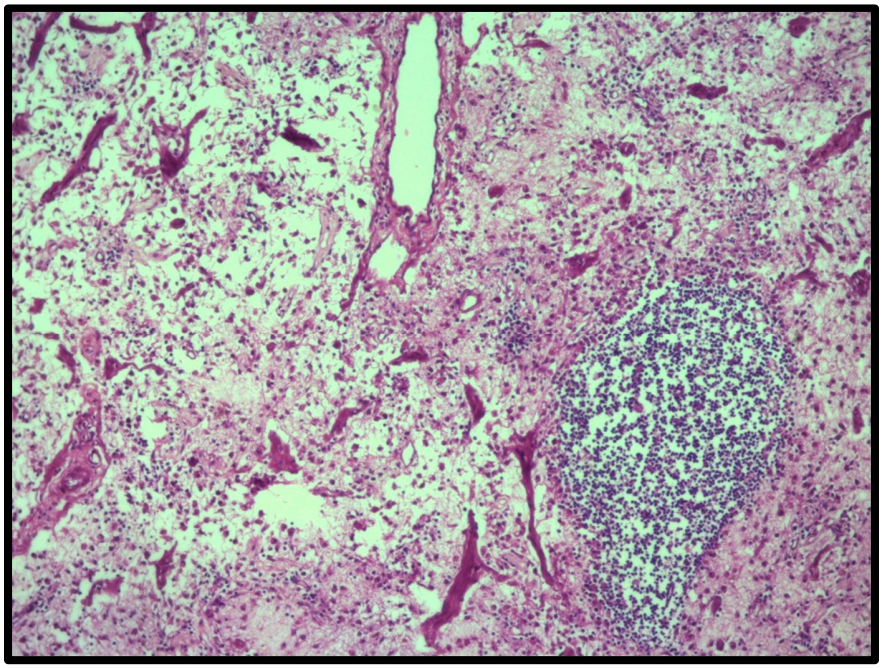

Plate 13: Section of lymph node showing oedema and depletion of lymphocytes. (H&E 100X)

enlargement of the draining lymph nodes due to proliferation of both infected and non-infected T- cells (Ahmad *et al.,* 2007). These two possible explanations for development of these findings are supported by the microscopical examination of Giemsa blood stained smear which showed presence of schizont forms of the *Theileria* parasites within many lymphocytes. Further, the intense hyperplasia of lymphocytes at the lymphopoietic centers is followed by the depletion and degeneration of these centers (Gill *et al.,* 1977).

4.4.2.3 Abomasum

Abomasum ulceration showed desquamation of the mucosal epithelial lining and there was congestion and infiltration of mononuclear cells in the submucosa (Plate 15). These findings are in accordance with Omer *et al*., (2002), Aparna *et al.,* (2011), Panda *et al.,* (2011), Acharya P., (2016) and Gupta *et al*., (2016).

4.4.2.4 Lung

Lung revealed congestion, alveolar emphysema and bronchial hyperplasia (Plate 16, 17). Alveoli were filled with inflammatory exudates leading to pneumonia (Plate 18) in buffaloes found to be positive for *Theileria*. These findings are in accordance with Panda *et al.,* (2011), who noticed emphysema, thickened alveolar wall due to congestion or cellular proliferation, pneumonic changes, congestion, fibrinous and haemorrhagic infiltration into the alveoli in cattle died due to Theileriosis. Similarly, Acharya (2016) also found congestion, haemorrhages and infiltration of inflammatory exudates in alveolar lumen. Further, Omer *et al*., (2003) also reported degeneration and lymphocytic infiltration in lungs.

The pulmonary lesions in the present study are described cells infected with *Theileria* schizonts induce polyclonal naïve T lymphocyte proliferation (Cambell *et al.,* 1995) which switches to a T helper 1 phenotype, producing large quantities of IFN-y together with excessive production of pro-inflammatory cytokines (including IL-1α, IL-1β, IL-6 and TNF- α) are probably the main cause of parasite inducing the pathological lesions (Brown 1990 and Glassa *et al.,* 2003)

4.4.2.5 Liver

Liver revealed dissociation of hepatocytes, severe congestion of sinusoidal vessels and increased sinusoidal space. The proliferation of fibrous

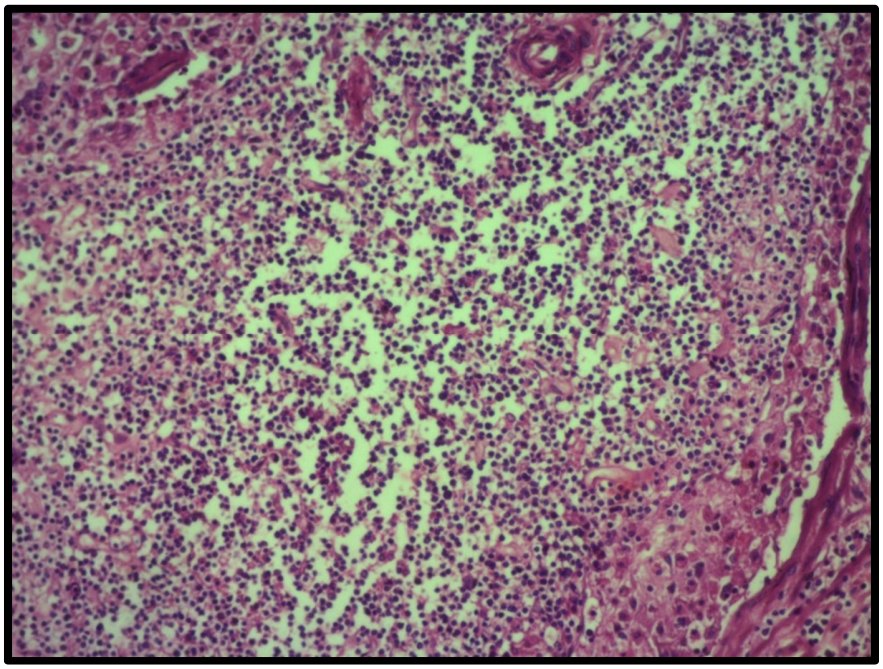

Plate 14: Section of lymph node showing depletion of lymphocytes.

(H&E 200X)

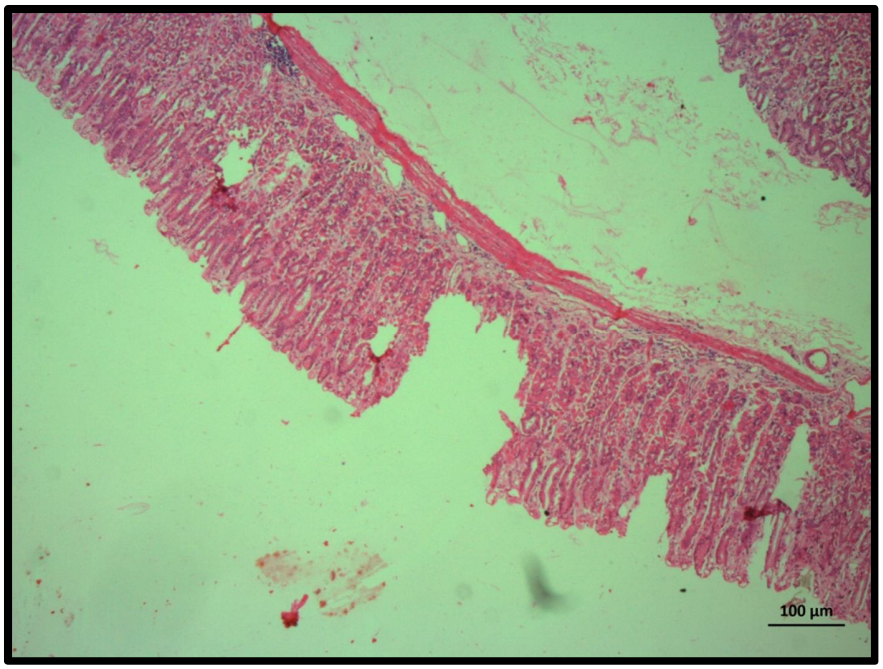

Plate 15: section of abomasum showing desquamation of epithelium and ulcer

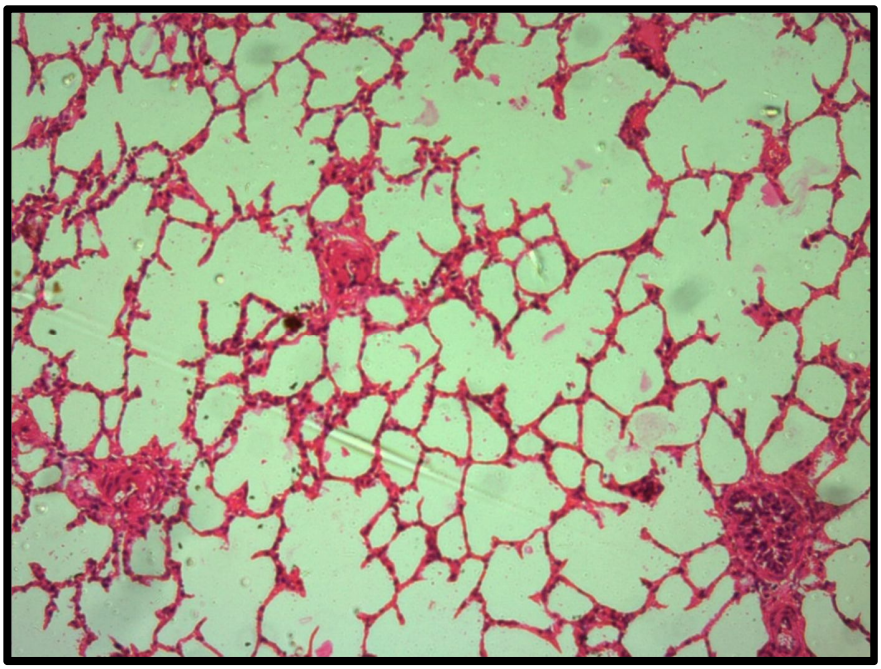

Plate 16: Section of lung showing emphysema and congestion. (H& E 100X)

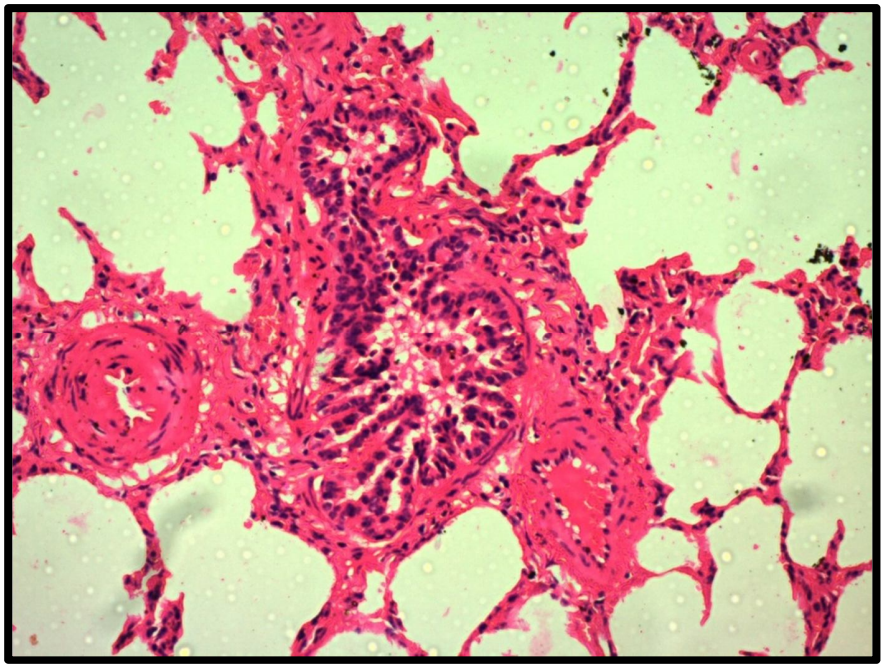

Plate 17: Section of lung showing emphysema and bronchial hyperplasia. (H& E 400X)

connective tissue in portal triad of liver was also evident in the *Theileria* positive buffaloes (Plate 19). These findings are in agreement with Panda *et al.,* (2011), who also noticed fibrosis of the portal tract and perivascular area in tissue section of liver of cattle died due to Theileriosis and Aparna *et al.,* (2011) as they observed periportal sinusoidal dilatation and proliferation of lymphocytes over the portal triad in cross bred bovines found positive for *T. orientalis*. However, Acharya (2016) observed congestion, haemorrhage, necrosis in areas surrounding to central veins, whereas Omer *et al*., (2002) reported fatty changes in the section of liver of cattle died to *T. annulata* infection.

4.4.2.6 Kidney

Renal changes were glomerular congestion and vacuolar degenerative changes in the tubular epithelium. (Plate 20). In some cases, occlusion of tubules and severe haemorrhages in intertubular space was also evident in *Theileria* positive buffaloes (Plate 21, 22). These findings are in agreement with Acharya (2016) who noticed degeneration, necrosis and sloughing of the tubular epithelium due to Theileriosis. Further, Panda *et al.,* (2011) also observed degeneration and necrosis involving all parts of the kidney.

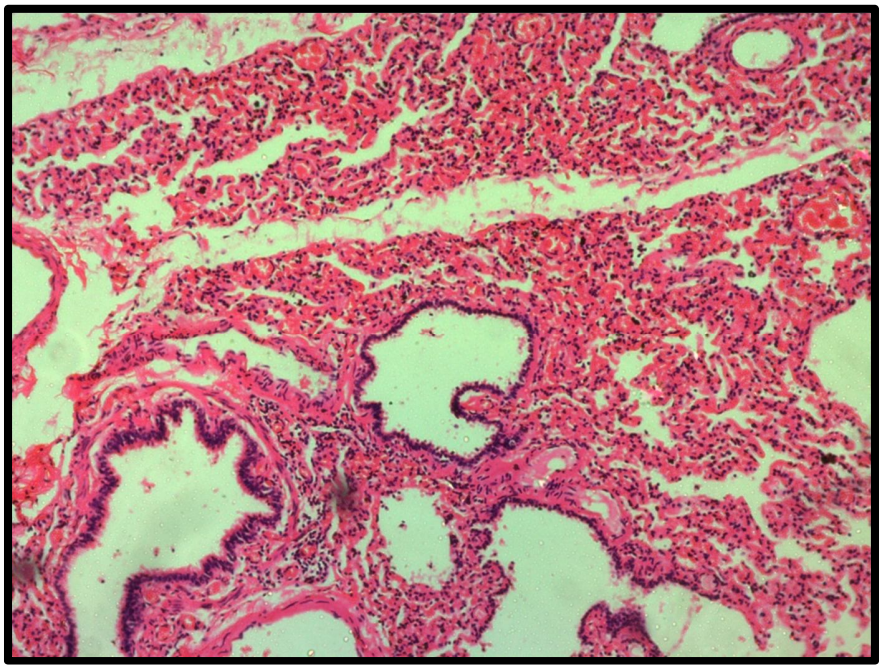

Plate 18: Section of lung showing alveoli filled with inflammatory exudates leading to pneumonia (H& E 400X)

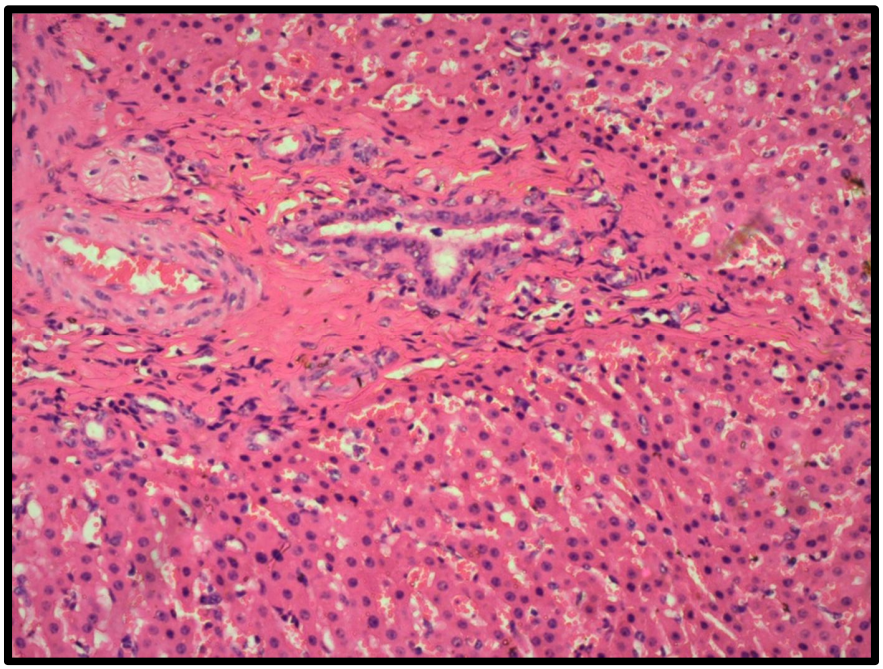

Plate 19: Section of liver showing proliferation of fibrous connective tissue in portal triad. (H& E 200X)

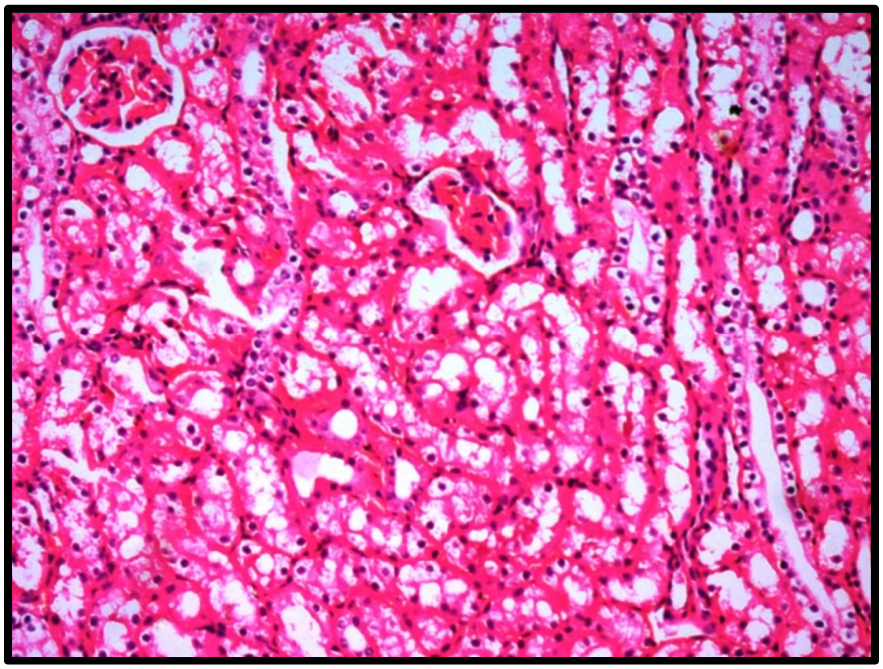

Plate 20: Section of kidney showing glomerular congestion and tubular vacuolar degenerative changes. (H& E 400X)

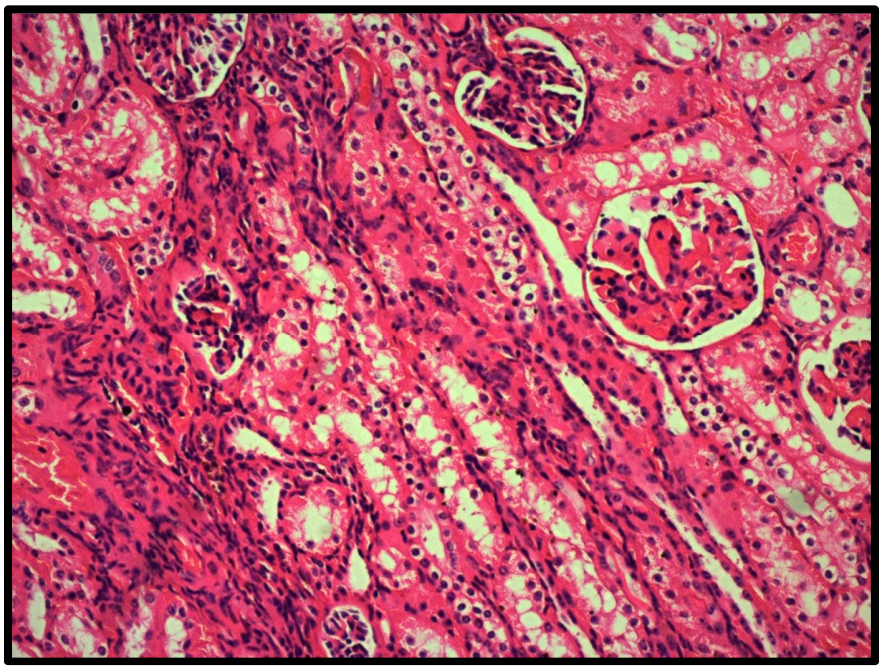

Plate 21: Section of kidney showing glomerular congestion and occlusion of tubules.

(H& E 200X)

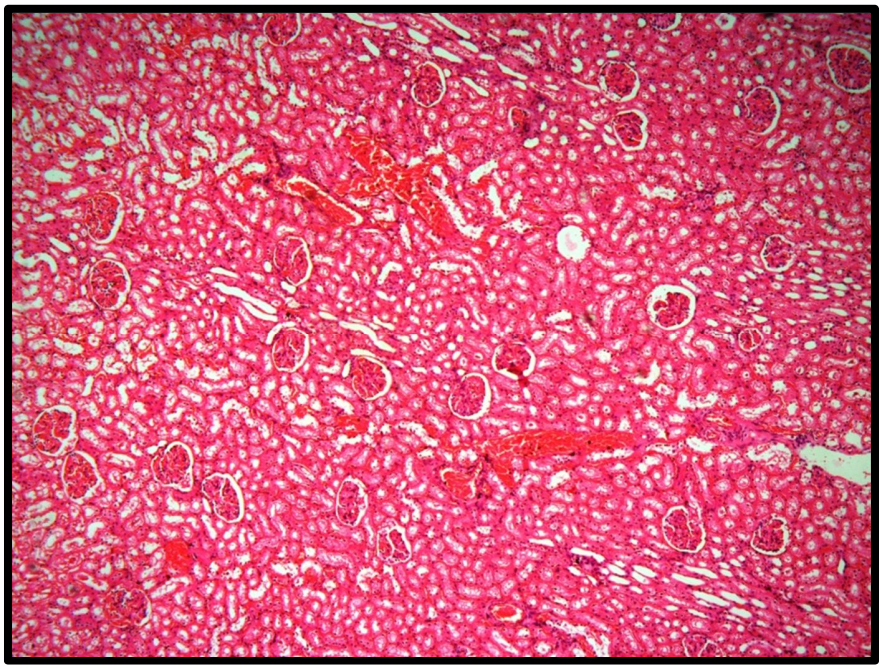

Plate 22: Section of kidney showing glomerular congestion and hemorrhages in intertubular space. (H& E 50X)

SUMMARY AND CONCLUSIONS

Tropical Theileriosis causes direct loss by mortality and indirect loss due to infertility, loss of productivity and high cost of treatment in bovines leading to severe economic losses.

Field diagnosis of acute Theileriosis is based on clinical signs and detection of schizonts in lymphocytes or monocytes and piroplasms in erythrocytes in Leishman's or Geimsa stained blood smear having certain limitations in diagnosis of chronic or subclinical cases. Hence, present research work was carried out to study pathology and diagnosis of *Theileria* infection in buffaloes.

The present study was carried out in Department of Veterinary Pathology, Veterinary Parasitology and TVCC of Nagpur Veterinary College, Nagpur. Blood samples from ear and jugular vein of 227 *Theileria* suspected buffaloes from Nagpur region were collected to study haematobiochemical alterations and to detect the *T. annulata* and *T. orientalis* by PCR assay.

From the microscopic examination of Leishman's or Geimsa stained blood smear, total of 40 blood sample were found positive for *Theileria*, out of which 13 blood samples were collected from jugular vein and 27 from ear vein.17.62% prevalence of Theileriosis was noticed on the basis of microscopic examination of blood smear. The sensitivity of blood sample collected from jugular and ear vein was found to be 28.88% and 31.39 % respectively while specificity for both was 100% indicating ear vein was best site for microscopic diagnosis of Theileriosis in buffaloes.

The piroplasms in the erythrocytes and macro or microschizonts in lymphocytes (Koch's blue bodies) and monocytes in stained blood smears of *Theileria* infected buffaloes were noticed. Anisocytosis, microcytosis and hypochromic erythrocytes were also noticed in *Theileria* infected buffalo indicating mild to moderate microcytic hypochromic anaemia.

For molecular diagnosis of *T. annulata*, TAMS1 gene was detected by amplifying 846 bp gene fragment using specific primers. Similarly, for *T. orientalis*, MPSP gene was detected by amplifying 852 bp gene fragment by using specific primers. An expected amplified product of 846bp of TAMS1 gene

for confirmation of *T. annulata* was obtained in 69 blood samples, Out of which, 33 blood sample were collected from jugular vein and 67 from ear vein. Similarly, for diagnosis of *T. orientalis*, an amplified product of 852 bp of MPSP gene was obtained in total of 13 blood samples. Out of which, 8 samples were collected from jugular vein and 12 from ear vein. In addition to this, a total of 7 samples found positive for both *Theileria annulata* and *Theileria orientalis* (4 from jugular and 7 from ear vein).

On the basis of PCR assay, prevalence of Theileriosis in buffaloes of Nagpur region was revealed as 39.20 % (89/227). However, prevalence of *T. annulata*, *T. orientalis* and both (*T. annulata* and *T. orientalis)* infections in buffaloes was found to be 30.39% (69/227), 5.72% (13/227) and 3.08% (7/227), respectively.

Haematological parameters revealed significant by decreased values of Hb, TEC, PCV, MCV, MCH, MCHC in buffaloes positive for *T. annulata* and both (*T. annulata* and *T. orientalis)* infection indicating microcytic hypochromic anaemia. In addition of these values were found to be non-significantly lowered in *T. orientalis* positive buffaloes. The mean TLC was significantly decreased in all the *Theileria* positive buffaloes. Mean of lymphocytes count was found to be increased in buffaloes positive for both *T. annulata* and *T. orientalis* followed by *T. annulata* and *T. orientalis* infection.

Serum biochemical profiles revealed significant decrease in values of total protein and albumin in buffaloes found positive for both *T. annulata* and *T. orientalis* followed by *T. annulata* infection. SGOT and SGPT levels were significantly increased in all the *Theileria* infected buffaloes as compared to the healthy buffaloes.

Post mortem examination of six buffaloes suspected to have died due to *Theileria* spp. infection was conducted for study of gross and histopathological lesions. The severities of lesions were more pronounced in buffaloes which were positive for both *Theileria* spp. followed by *T. annulata* and *T. orientalis* infection.

Gross lesions revealed frothy tracheal content, congestion, emphysematous and pneumonic changes in lungs. Pre-scapular lymph nodes

and spleen were enlarged. In all the cases, abomasum showed punched out haemorrhagic ulcers. Gelatinisation of peri renal fat and pale areas in kidney were observed. Histopathological examination of prescapular lymph node and spleen revealed depletion of lymphocytes. Moderate to severe loss of mucosal epithelium with desquamation and necrosis of mucosal and submucosal tissue were observed in abomasum. Lung showed pneumonia, alveolar emphysema and bronchial hyperplasia. Liver showed congestion in sinusoidal space and proliferation of fibrous connective tissue in portal triad. Kidneys revealed vacuolar degenerative changes, occlusion of tubules and severe hemorrhages in intertubular space.

Conclusions:

1. On the basis of microscopic examination of stained blood smear the prevalence of Theileriosis was found to be 17.62% (40/227). The sensitivity of blood sample for diagnosis of Theileriosis collected from jugular and ear vein was found to be 28.88% and 31.39 %, respectively.

2. On the basis of PCR assay, overall prevalence of Theileriosis in buffaloes of Nagpur region was 39.20 % (89/227). However, prevalence of *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* in buffaloes was found to be 30.39% (69/227), 5.72% (13/227) and 3.08% (7/227) respectively.

3. Haematobiochemical alterations and pathomorphological changes were more pronounced in buffaloes found positive for both *T. annulata* and *T. orientalis* followed by *T. annulata* and *T. orientalis* infection.

4. Ear vein was found to be the best site for collection blood samples diagnosis of Theileriosis.

5. PCR technique was found to be the highly specific and sensitive for the diagnosis of Theileriosis at any stage of disease.

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APPENDIX

1. Composition of RBC lysis buffer for DNA extraction

NaCl	:0.22 %
Saponin	:0.015 %
EDTA	:1 mM
pH 7.5	

2. Digestionbuffer for DNA extraction

KCl	:50 mM
Tris-HCl	:10 mM
Tween 20	:0.5 %
Proteinase K	:100 µg
pH 8.0	

3. Tris Borate EDTA buffer (TBE) (5x stock solution)

Tris Base /Tris buffer	:54.0gm
Boric acid	:27.5gm
0.5M EDTA	:20ml
Distil.Water	:1000

Stored at room temperature

For use dilute 1:100 with water for Agarose Gel Electrophoresis

4. Proteinase K (20 mg/ml)

Proteinase K	: 20 mg
Distil. Water make upto	: 1ml

5. Ethidium bromide solution

Ethidium bromide	: 0.1gm
Distil. Water make upto	: 10ml

Reagents for haematological examination

1. V-28CFL LYSE
2. V-28R RINSE
3. V-28D DILUENT

Reagents for Biochemical Examination

I. Total protein kit
Carton 1
L1 Biuret Reagent
L2 Protein Standard (8g/dl)

II. Albumin Kit
Carton 1
L1 BCG Reagent
Carton 2
Albumin Standard (4g/dl)

III. SGPT Kit
R1: 3* 40 ml
R2: 3*10 ml

IV. SGOT Kit
R1: 3* 40 ml
R2: 3*10 ml

VITA

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THESIS ABSTRACT

a)	Title of thesis	:	" **Pathology and diagnosis of *Theileria annulata* and *Theileria orientalis* in buffaloes*"***
b)	Full name of student	:	**INGLE PATIL PRATIK NARAYANRAO**
c)	Name and address of advisor/Guide	:	**DR. P. M. SONKUSALE** Assistant Professor Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur.
d)	Degree to be awarded	:	MASTER OF VETERINARY SCIENCE
e)	Year of award of degree	:	2017
f)	Major subject	:	**VETERINARY PATHOLOGY**
g)	Total number of pages in the thesis	:	44
h)	Number of words in the thesis abstract	:	440
i)	Signature of student	:	
j)	Signature, name & Address of forwarding authority	:	

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ABSTRACT

Tropical theileriosis is economically important disease of bovines, which is routinely diagnosed by microscopic examination of stained blood smear for presence of schizonts in lymphocytes or monocytes and piroplasms in erythrocytes, Although this method is widely used for laboratory based diagnosis, however it is combined to have certain diagnostic limitations.Hence, the present

work entitled “Pathology and diagnosis of *T. annulata* and *T. orientalis* in buffaloes” was conducted to compare the specificity and sensitivity between haematological and PCR based method.

A total of 227 blood samples from ear and jugular vein were collected from T*heileria* suspected buffaloes from in and around Nagpur region to study haematobiochemical alterations and to detect the *T. annulata* and *T. orientalis* by PCR assay.

Microscopic examination of Leishman’s or Giemsa stained blood smear, showed a total of 40 blood sample positive for *Theileria*, out of which 13 were collected from jugular vein and 27 samples from ear vein. The prevalence based on blood smear examination was recorded as 17.62% was noticed on the basis of microscopic examination of blood smear. The sensitivity of blood sample collected from jugular, ear vein and both (jugular,ear vein) was found to be 28.88%,31.39 % and 100% respectively.

A total of 69 blood samples showed an expected amplified product of 846 bp of TAMS1 gene for confirmation of *T. annulata*. Out of which, 33 blood sample were collected from jugular vein and 67 from ear vein. For diagnosis of *T. orientalis*, an amplified product of 852 bp of MPSP gene was obtained from 13 blood samples, out of which, 8 samples were collected from jugular vein and 12 from ear vein. In addition 7 samples found to be positive for both,*Theileria annulata* and*Theileria orientalis* (4 from jugular and 7 from ear vein).

On the basis of PCR assay, prevalence of Theileriosis in buffaloes of Nagpur region rewarded as 39.20 % (89/227). However, prevalence of *T. annulata*, *T. orientalis* and both *T. annulata* and *T. orientalis* infection in buffaloes was found to be 30.39% (69/227), 5.72%, 13/227) and 3.083% (7/227), respectively.

Haematobiochemical and pathological changes were more pronounced in buffaloes positive for both *T. annulata* and *T. orientalis* followed by *T. annulata* and *T. orientalis* infection.

Gross lesions revealed enlargement of spleen and pre-scapular lymph nodes and punched out ulcers in abomasum. Histopathologically, depletion of lymphocytes in prescapular lymph node and spleen and necrosis, desquamation of abomasal mucosal epithelium, pneumonic change and fibro vascular change in hepatic parenchyma was observed.

The study can be concluded that the severity of disease was more pronounced in buffaloes which were positive for both *Theileria* spp. followed by *T.*

annulata and *T. orientalis* infection. Ear vein is the preferred site over the jugular vein for the collection of blood samples for diagnosis of Theileriosis. PCR technique is highly specific and sensitive for diagnosis of Theileriosis at any stage of the disease.

ic/kl kjk'k

v-	ic/kkp f'k"kd	%	^^Eg'khe/khy Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfylp jkxfunku vkf.k gk.kkÚ;k fodrhPkk vH;kl-**
c-	fo|kF;kp i.k uko	%	**bxG ikVhy ifrd ukjk;.kjko**
d-	ekxn'kdkp uko o iRrk	%	Mk- i'kkr lkudlG lgk;d ik/;kid fodrh 'kkL= foHkkx ukxij i'ko|d egkfo|ky;]ukxij
M-	inku dj.;kr ;.kkjh inoh	%	**Lukrdkrrj inoh ¼e-Ogh-,l-lh-½**
b-	inoh inku dj.;kp o"k	%	**2017**
Q-	e[; fo"k;	%	fodrh 'kkL=
x-	ic/kkrhy ,d.k i"B	%	**44**
g-	lkjk'kkrhy ,d.k 'kCn	%	**407**
b-	fo|kF;kph lgh	%	
t-	vxf"kr dj.kkÚ;k vf/kdkÚ;kph lgh] uko vkf.k iRrk	%	

¼Mk,u-,u->kM½
lg;kxh vf/k"Bkrk
ukxij i'ko\|d egkfo\|ky;]
ukxij

---

ic/kl kjk'k

m".k dVhcnh; Fkk;yfjvkfll gk xko'kh; ik.;ke/khy vkfFkd n"V;k egRokpk jkx vkg- T;kp funku jDr dkpiVVhpk l{e n'kh; vH;kl d:u jDrkrhy yfldk i'kh fdok ekukl k;bV e/khy lk;>kV rlp rkcM;k

i'khe/khy ikjklyk>epk mifLFkrh brdp e;kfnr vkgr- ;k jkxfunkukojhy e;knk ikgrk ;koj l'kk/ku dj.;kr vkyR;kp Lo:i ^^Eg'khe/khy Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfylp jkxfunku vkf.k gk.kkÚ;k fodrhPkk vH;kl-** vl gkr-

Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfylp funku dj.;klkBh jDrtho 'kkL=h;] ldG@BGd vkf.k mrhrhy fodrcnykpk vH;kl dj.;klkBh ih-lh-vkj i/nrhpk voyc dj.;kr vkyk- R;k dfjrk ukxij ifjljkrhy Fkk;yjh;k lf/kX/k 227 Eg'khp dku o ekurhy jDrokfgU;krhy jDrkp ueu ldfyr dj.;kr vky vkg-

yh'keu fdok thelkLVupk jDrdkpiVVhP;k l{en'kh; rikl.khru] Fkk;yjh;k dfjrk 40 jDrkp ueu ldkjkRed vk<Gy vlu] R;kidh ekuP;k f'kjrhy vkf.k dkUkkrhy f'kjru vude 13 o 27 jDr ueu ldkjkRed vk<Gy vkf.k iHkko 17-62 VDd fnlu vkyk- jDriVVhP;k l{enf'k; rikl.khP;k vk/kkjkojekuP;k vkf.k dkuP;k f'kjkrhy jDrueU;kph lonuk vude 28-88 vkf.k 31-39vk<Gyh vkf.k nkUgh pkp.kh fof'k"Brk gkrh-

;k Fkk;yjh;k vU;ykVkojhy [kk=hyk;d funkukdfjrk 69 VEl 1 tudkrhy vif{kr fo'kknh dj.kdj.;kr vkyY;k 846 fcih otukpk iVVk feGfo.;kr vkykR;krhy ekuP;k o dkukP;k f'kjrhy vude 33 o 68 ueu ldkjkRed feGky@vk<Gy R;kpiek.k ;k Fkk;yfj;k vkj,UVk;fylP;k funkukdfjrk ,e-ih-,l-ihtudkpk fo'kknhdj.k dY;k 852 ch-ih- iVVk 13 ueU;kru ikIrdj.;kr vkyk R;krhy 8 ueu gekuP;k f'kjru rj mofjr 12 ueu dkukP;k f'kjru ldfyr dj.;kr vky- rlp ,d.k 7 ueu nkUgh ¼Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfyl ½ dfjrk ldkjkRed vk<Gy ¼4 ueuekuP;k f'kjru rj 7 ueudkukP;k f'kjru½-

R;kpieku ihlhvkj i/nrhP;k fudkykP;k vk/kkjkoj Fkk;yfj;kP;k ukxij ifjljkrhy Eg'khoj 39-20 VDd ¼89@227½ ,o<k iHkko vlY;kp vk<Gy-rlp Eg'khe/khy ;k Fkk;yjh;k vU;ykVk] Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfyl nkUgh llxkP;k Fkk;yjh;k vkfj,UVfylpk iHkko vude 30-39

VDd ¼69@227½] 5-72 VDd ¼13@227½ vkf.k 3-083 ¼7@227½ vlY;kpvk<Gy-

Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfyl nkUgh dfjrk ldkjkRed vlyY;k eg'khe/; jDrtho 'kkL=h; o fodr 'kkL=h; cny vf/kd BGd vlY;kp vk<Gy- o R;k[kkyk[kky Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfyl ;k llxkr-

,d.k fodrheG o/nh vkf.k fiLdiY;vj yfldkxFkhph ok< vkf.k vcke>e e/; ip vkÅV vYlj fnlu vky- fgLVkiFkkykftdy] fiLdiY;vj yfldk xFkh vkf.k lyhgk e/khy ylhdk i'kh deh vkf.k tBjkP;k 'y'ey Roppk dkgh Hkkx u"V gk.k] vLrj i'kh legkpk Úgkl gk.k] 'olu nk"k vkf.k ikVy f=dVP;k i'khprre;hdj.k y{kkrvky-

Eg.ku fu"d"k dk<yk tkÅ 'kdrk dh Eg'khe/; jkxkph rhork vf/kd Li"V nkUgh ¼Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfyl½ldkjkRed gkrh-R;kurj Fkk;yjh;k vU;ykVk o Fkk;yjh;k vkfj,UVfyl ldeukr vk<Gy- Fkk;ykfj;kfllP;k funkuklkBh dkukph f'kj g lokRre tkxk vkg- jkxkP;k dk.kR;kgh VII;koj Fkk;ykfj;kfllPk funku dj.;klkBh ih lh vkj r= vR;r fof'k"V vkf.k lonu f'ky vkg-