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Changes in haemato-biochemical profiles in acutely infected bovine with tropical theileriosis

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Abstract
The present study was carried out on blood samples of 33 acutely infected cattle with theileriosis collected from four different agroclimatic zones of Bihar. These samples were screened out based on clinical signs and symptoms, presence or history of tick infestation and found positive under microscopic examination as well as through PCR. The sample were processed and analyzed. Hematological parameter under study comprises Hemoglobin percent (Hb%), Total Erythrocyte Count (TEC), Platelets count, Total Leucocytes Count (TLC), Differential Leucocytes Count (DLC) Biochemical profiles was Total plasma protein (TPP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Tumor Necrosis Factor-α (TNF-α). Marked fall in Hb% (p-value: <0.001), Erythrocyte Count (p-Value: <0.001), WBC count (p-value: <0.001) and platelets (p-value: <0.001) were noticed. In Differential leucocytes count, significant decline in lymphocytes (p-value: <0.00001), neutrophil (p-value: <0.00001), Eosinophils (p-value: <0.00001) were found without any noticeable change in monocytes (p-value: 0.268) and Basophil counts (p-value: 0.951). Significant rise in Serum ALT/SGPT (p-value <0.001), AST/SGOT (p-value: <0.001) and TNF-α (p-value: 0.001) were found whereas marked decrease in Total Plasma Protein (TPP) (p-value: <0.001) was noticed.

Keywords: Theileriosis, bovine, hematological profile, biochemical profiles

Introduction
Tropical bovine theileriosis, tick borne haemo-protozoan disease, is of great concern in bovine as it causes severe economic losses to livestock farmers across the world due to high incidence of mortality, weight losses, abortions, reduced milk yield and control measures (Gharbi *et al.*, 2006, 2011) [12, 13]. A global figure for the economic losses and importance of *T. annulata* is not available, but this parasite is accepted to affect the productivity of perhaps 250 million cattle, causing morbidity in indigenous cattle and mortality of between 40-60% 'improved' cross-bred or exotic animals (Brown, 1990) [6]. The economic impact of *T. annulata* in India was estimated to be US$ 800m, based on direct losses due to mortality and production losses (Milk yield, growth rate, meat, infertility, abortion, calving interval and hides) and the indirect costs of control measures (dipping, vaccination, chemotherapy, veterinary legislation and monitoring) (Brown, 1997) [7]. The causative agent, *Theileria annulata*, is found to be widely distributed in both tropical and sub-tropical region and most profoundly occurs around the Mediterranean basin, Middle East and Southern Asia (Gubbels *et al.*, 1999; Garcia-Sanmartin *et al.*, 2006; Branco *et al.*, 2010; Silva *et al.*, 2010) [15, 11, 5, 27].
Life-cycle commences with inoculation of mature sporozoites by ticks belongs to genus *Hyalomma* spp. during engorgement leads to massive lymphoproliferation in sequential events initiated by transformed macro-schizonts infected cells. These macro schizonts infected cells on further development precede microschizont-infected cells which in due course give rise to merozoites. These merozoites in turn become intra-erythrocytic.
During the infection, several haemato-biochemical profiles must be altered due to marked dysfunction of the lymphoid and reticuloendothelial cells. In such condition, it is of utmost important to understand the changes in haemato-biochemical parameter in acutely infected animals which may become the ultimate tools for diagnosis, prognosis, and therapy. It may also assist to understand the severity of infection. Therefore, in the present study, changes in these parameters were investigated to further understand the pathogenesis of tropical theileriosis.

Materials and Methods

Strategies for collection of samples from different agro-climatic zones of Bihar

Study was performed in phase wise manner covering all the four zones of Bihar. Sampling was conducted in one district each of all the four zones include Muzaffarpur, Araria, Patna and Lakhisarai which comes under zone I, II, IIIA and IIIB respectively (Fig. 1).

A total number of 400 samples were collected for the study including 100 samples from each district out of which a total number of 33 sample diagnosed positive and found acutely infected were selected for the present study and compared with equal number of samples randomly selected form healthy ones. The screening of these samples was made on the basis of clinical sign and symptoms, presence of tick infestation and presence of piroplasmic stage under microscopic examination which was finally confirmed by using specific genetic marker in PCR. Samples taken for the study were whole venous blood.

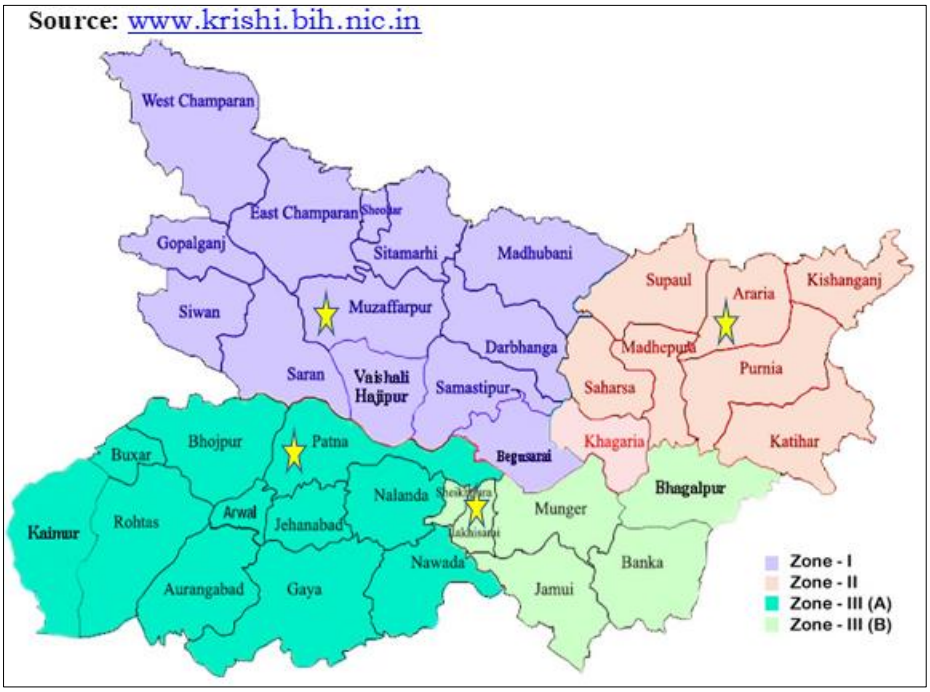


Fig 1: Districts selected (☆) for sampling from different agro-climatic zones of Bihar

Collection and processing of blood samples

Blood samples, approx. 6ml were collected aseptically from jugular vein of suspected animals and kept in two separate 2ml vial containing EDTA and rest was collected in another 2ml vial without EDTA. Samples collected in EDTA were kept in refrigerator under 4 °C and used for DNA extraction and hematological analysis. For biochemical analysis, blood sample collected in vial without EDTA was centrifuged at 2000xg for 10 min. Extracted plasma were collected and kept in another sterilized vial, duly marked, and stored at -70 °C.

Microscopic identification of piroplasmic stage of *Theileria* spp

Three thin blood smears from each sample were prepared and fixed in methanol for 5 min and stained with Geimsa stain (1:10) dilution for 30 min (Benjamin, 1978) [3] and examined under oil immersion objective lens for confirmation of piroplasmic stage.

Molecular confirmation of *Theilaria annulata*

DNA isolation were performed using commercial kit (GSure® Blood DNA Mini Kit, GCC Biotech.) and as per method described by manufacturer. Briefly, about 300 µl blood were used for extraction of genomic DNA. Isolated DNA was eluted in 50 µl nuclease free water. Confirmations of extracted DNA were made by running 1.5% agarose gel electrophoresis. Further, both quantitative as well as their purity were determined using spectrophotometer (Genova Nano; GENWAY) at 260 and 280 nm.

The genomic DNA was subjected to PCR for identification of the *T. annulata* using Tams 1F primer (5′ATGCTGCAAATGAGGAT3′) and *T. spms* 1 Rprimer (5′GGACTGATGAGAAGACGATGAG3′) for the amplification of 785 bp fragment of 30KDa major merozoite surface antigen genes (Kirvar *et al.*, 2000) [31]. Briefly, for amplification, the PCR reactions were set up in 25 µl volume containing 12.5 µl of Master mix (0.05/ml Taq DNA polymerase in reaction buffer, 4 mMMgCl2, 0.4mMdATP, 0.4 mMdCTP, 0.4 mMdGTP, and 0.4 mM dTTP), 2.0 µl of each primer and 2 µl of the extracted DNA template, and the total volume was made up to 25 µl using nuclease-free water. Conditions for PCR amplifications in automated thermo-cycler (Veriti 96 well Thermal Cycler) were standardized and the cycling conditions were 95 °C for 4 min followed by 35 cycles at 95 °C for 40sec, 54 °C for 50sec and 72 °C for 1 min. A final extension step of 70 °C for 10 min was also used. The PCR products of expected sizes were confirmed by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet trans-illuminator (Gel Doc 150; Azure Biosystem). Positive (targeted pathogen positive DNA) and negative controls were run along with each series of amplifications. A100-bp DNA ladder were used to initially determine the molecular mass of PCR products. The rest of the PCR product was kept at -20 °C until further use.

Haematology

Under haematological analysis collected blood samples were processed to evaluate Hb%, Total Erythrocyte Count, Total Leucocyte Count and Differential leucocytes count. Hb% were estimated in automatic analyzer (Hemocue® Hb 201 system). Briefly, 10 µl of gently mixed blood were charged

over micro cuvette and reading was taken by inserting it inside the Haemocue hb201 DMX analyzer. Total Erythrocyte Count, Total leucocyte counts and Platelet counts were performed in Hemocytometer (Neubauer chamber) using Grower's solution, W.B.C. diluting fluid and Reece -Ecker fluid as diluent for erythrocytes, leucocytes and platelets respectively. For counting Erythrocytes and leucocytes, protocols of Benjamin (2001) [4] were followed whereas for platelets counting methods of Jain (1993) [16] were used. For Differential leucocyte count, blood smears were prepared immediately after collection of blood samples, air dried and stained with Leishman's stain. Differential Leucocyte count was done as per Benjamin (2001) [4] and results were expressed in percent.

Biochemical estimation

Four important parameters *viz.* Alanine amino Transferase (ALT)/Serum Glutamic-Pyruvic Transaminase (SGPT), Aspartate Amino Transferase (AST)/Serum glutamic-oxaloacetate transaminase (SGOT), Total Plasma Protein (TPP) and Tumor Necrosis Factor-Alpha (TNF-α) were taken into consideration. For estimation of these parameters, commercially available kits and their protocols were followed. For quantification of SGPT, SGOT and TPP, instruction provided by Coral, India in their respective kits were used whereas for TNF-α, methods provided by G-Bioscience, USA, in Immuno-Tag, Bovine TNF-α, ELISA KIT was used.

Statistical Analysis

All the statistical analysis was performed in Sigma Plot Version 12. The results obtained are expressed as mean ± SE. For comparing the means of the groups of infected and non-infected groups for changes in the hemato-biochemical profiles during theileriosis, Student's t-test was used. Statements of statistical significance are based on $p<0.05$.

Results

Major clinical signs and symptoms found during collection of samples assume to be acute was marked rise in fever 40-41 °C, enlargement of superficial lymph node especially the pre-scapular lymph node (Fig. 2a), accelerated pulse rate, nasolacrimal discharge, inappetence, icteric conjunctiva. Heavy tick infestation was also found. (Fig. 2b & 2c). On microscopic examination of Giemsa-stained blood smear under 1000x, presence of piroplasmic stage in blood (Fig. 3) confirms the positive case. Presence of *Theileria annulata* in blood samples were finally confirmed through *PCR*. Amplicon size of 785bp found during running in 1.5% agarose gel electrophoresis confirm the species of the genus (Fig-4).

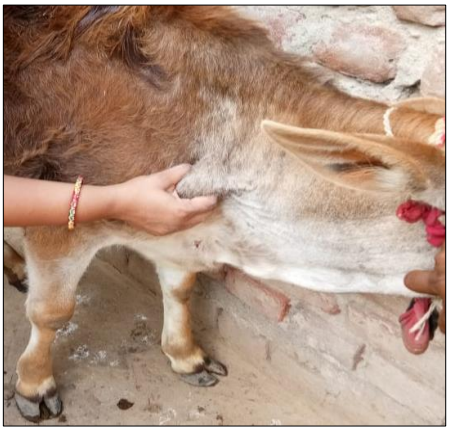


Fig 2a: Image showing pre-scapular lymph node swelling in calf

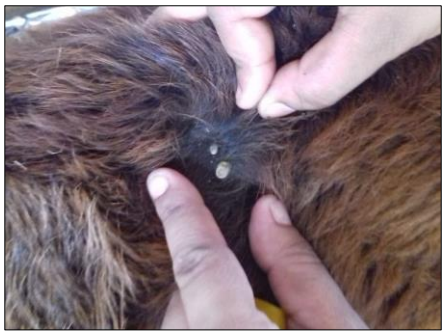


Fig 2b: Image showing cattle with tick infestation

Fig 2c: Image of *Hyalomma* spp. (Arrowed) and *Rhiphicephalus* (*Boophilus*) Spp.

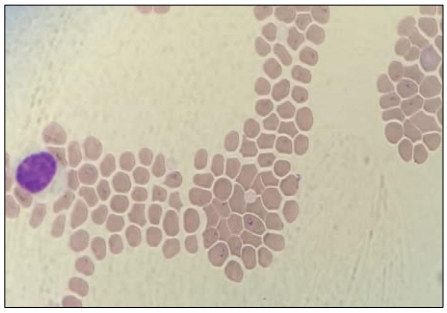
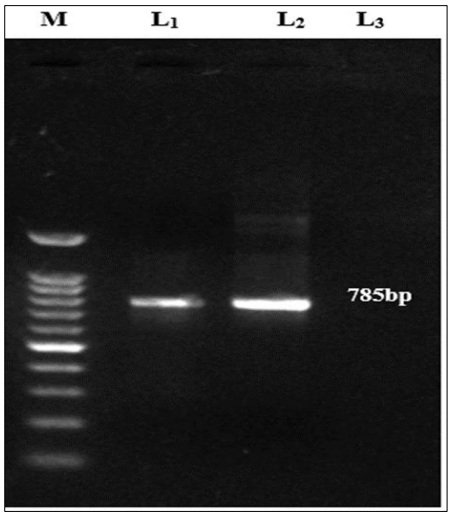


Fig 3: Image showing piroplasmic stage of *Theileria* spp. (1000x)



L_1 & L_3: Control positive and Control negative for *Theileria annulata* respectively
L_1 & L_2: band with 785bp confirming *Theileria annulata* Marker (M): 100bp DNA ladder

Fig 4: 1.5% Agarose gel electrophoresis confirming *Theileria annulata* using species specific primer Tams-1 (Forward) and Tspms-1 (Reverse)

Changes in haematological profiles in actute cases of theileriosis

By understanding the trends of rise and fall of different haematological parameter better understanding can be made for the assessment of the disease. For evaluating the relationship associated with the theileriosis and haematological changes, total 33 numbers of acutely infected samples were compared by equal number of randomly selected normal healthy animals (Table No. 01). Marked fall in haemoglobin per cent, Erythrocyte count, W.B.C. count and platelets were noticed (p-value-<0.001). In differential leucocytes count significant decline in lymphocytes (p-value-<0.00001), neutrophils (p-value-<0.00001) and eosinophils (p-value-<0.00001) were found without any noticeable changes in monocyte (p-value: 0.268) and basophils count (p-value-<0.951).

Table 1: Comparative hematological changes occurring in cows infected with *Theileria annulata* (Mean±SE)

Parameters	Unit	Non-infected cattle (n=33)	Infected cattle (n=33)	p-value
Haemoglobin	g/dl	10.65±0.15	5.52±0.067	<0.001
Erythrocytes (R.B.C)	10^6/mm^3	7.82±0.07	3.50±0.37	<0.001
Leucocytes (W.B.C)	/mm^3	9168.18±134.90	5027.27±129.13	<0.001
Lymphocyte (Absolute)	/mm^3	5368.18±83.39	2846.85±86.29	<0.00001
Neutrophils (Absolute)	/mm^3	3127.03±74.09	1812.27±62.49	<0.00001
Monocyte (Absolute)	/mm^3	216.06±7.91	204.98±6.26	0.268
Eosinophils (Absolute)	/mm^3	428.83±23.10	138.30±5.57	<0.00001
Basophils (Absolute)	/mm^3	25.39±7.46	24.86±4.42	0.9506
Platelets	10^5/mm^3	4.28±0.12	1.87±0.073	<0.001

Biochemical changes in acutely infected animals

Various components normally present in serum become altered during theileriosis (Table No. 02). Severity can be assessed by interpreting those altered components. Two important diagnostic enzymes *viz.* ALT/SGPT, AST/SGOT and other important parameters *i.e.,* total plasma protein (TPP) and Tumor necrosis factor-α (TNF-α) were also evaluated during acute phase of theileriosis in comparison to normal healthy animals (Table: 02). Significant rise in serum ALT/SGPT (p-value-<0.001), AST/SGOT (p-value-<0.001) and TNF-α (p-value-<0.001) were found whereas marked decrease in TPP (p-value-<0.001) were noticed.

Table 2: Comparative biochemical changes occurring in cows infected with *Theileria annulata* (Mean±SE)

Parameters	Unit	Non-infected cattle (n=33)	Infected cattle (n=33)	p-value
Total Protein	g/dl	6.375±0.0495	4.267±0.115	<0.001
SGPT/ALT	Units/ml	19.455±1.021	45.027±1.188	<0.001
SGOT/AST	Units/ml	80.824±1.685	134.485±1.794	<0.001
TNF-α	ng/L	176.390±6.285	807.476±65.320	<0.001

Discussion

Significant changes found above in haematological parameter were in accordance with Aulakh and Singla (2006); Radostitis *et al*. (2007); Durrani and Kamal (2008); Vahora *et al*. (2009); Masare *et al*. (2009); Ananda *et al*., (2009) and Qayyum *et al*. (2010) [2, 23, 10, 30, 17, 1, 22]. In differential leucocytes count significant decline in lymphocytes (p-value-<0.00001), neutrophils (p-value-<0.00001) and eosinophils (p-value-<0.00001) were found without any noticeable changes in monocyte (p-value: <0.268) and basophils count (p-value-0.951). Omer *et al.* (2002) [20], during his study made over 41 Holstein Friesian cows infected with theileriosis had shown the similar change in blood parameter with similar impact. These changes in blood parameter were also reported by Godderis (2004) [14], in claves carrying *T. annulata* infection in Kenya. Changes in these blood parameters in diseased animals are the prime indication of occurrence of severe anemia, panleukopenia, lymphocytopenia, eosinopenia and neutropenia.

Significant rise in serum ALT/SGPT (p-value-<0.001), AST/SGOT (p-value-<0.001) and TNF-α (p-value-<0.001) were found whereas marked decrease in TPP (p-value-<0.001) were noticed. Similar changes in the serum ALT/SGPT and AST/SGOT were also reported by Murray *et al.* (1990) [18] confirming the necrosis of liver. Sandhu *et al.* (1998) [25], describes the reason for rise in these enzymes. According to them, it happens due to hypoxia resulting from anaemia and jaundice. *Theileria annulata* infection leads to hepatic tissue damage include coagulative necrosis, distortion of hepatic cords and heavy infiltration of lymphocytes in peri-portal areas, indicating severe damage to the hepato-biliary system. Similar finding was also reported by Col and Uslu (2007); Saber *et al.* (2008) and Ugalmugle *et al*. (2010) [9, 24, 29]. Significant rise in TNF-α were also reported by Brown *et al.* (2008) [8] during theileriosis. Cytokines play a crucial role produced by *T. annulata* infected cells during progression of disease. These infected cells constitutively produce several macrophages associated cytokines, including IL-1α, IL1-β, IL-6, IL-10 and TNF-α inducing high level of T-cell proliferation. Among them TNF-α and inflammatory cytokines is potent inducer of fever and has also been found linked to the production of anemia, muscle wasting and necrosis (Ohmann *et al*., 1989 and Sileghem *et al*., 1994) [19, 26]. These elevated TNF-α linked symptom are mostly seen in acute stage. Several time it has been seen that animal death occurs before detection of piroplasm. Pipano and Isreal (1971) [21] explained that death in these animals is directly linked with rise in TNF-α during infection and it is not the piroplasmic stage where lysis of erythrocytes leads to anemia and fever. Brown *et al.* (2008) [8] suggests that the production of TNF-α directly causes the pathological reactions observed during *T. annulata* infections.

The marked decrease in total serum protein during theileriosis was noticed. Singh *et al.* (2001) [28] and Saber *et al.* (2008) [24] observed the hypoproteinemia and hypoalbuminemia in *Theileria* infected cattle. According to them, decrease in TPP might be due to harmful effects of toxic metabolites of *Theileria* and due to liver failure.

Conclusion

In theileriosis, the acutely infected animals show significant decrease in haemoglobin per cent, Total erythrocyte count and W.B.C count. There is marked fall in Lymphocyte, Neutrophils and Eosinophils. Rise in ALT/SGPT, AST/SGOT and TNF-α with significant fall in total serum protein may happen. Analyzing these changes in acute condition may help us in diagnosis, prognosis as well as in therapy.

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