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Comparative haemato-biochemical alteration in theileriosis and babesiosis detected by duplex PCR in cattle

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ABSTRACT

Blood samples from suspected crossbred cows (327) were examined microscopically as well as confirmed by in-house standardized duplex PCR assay. Out of 327 samples, 107 (32.72%) and 17 (5.19%) samples were positive for *T. annulata* and *B. bigemina* respectively by microscopy. When the samples were screened by duplex PCR, 130 (39.75%) and 27 (8.25%) animals had single infection with *T. annulata* (Group I) and *B. bigemina* (Group II), respectively. Duplex PCR was able to detect 11% of mixed infections (Group III) compared to 2.75% by microscopy. Haemato-biochemical profile of infected animals (30 for each group) were studied and compared with each other and normal healthy group (Group IV, n=10). The infected group showed significantly decreased levels of TEC, Hb and PCV, red blood cell indices than healthy control animals indicating microcytic hypochromic anaemia. Marked thrombocytopenia was also observed in affected animals. Serum biochemistry of infected cows revealed significantly higher values of AST and low levels of blood glucose, calcium, total protein, albumin as compared to healthy animals. Group I and Group II were further sub-divided into three sub-groups based on severity of infection as latent, subclinical and clinical. No significant difference among biochemical parameters was observed between subgroups of diseased animals but there was significant decline in hematological parameters, viz. haemaoglobin, PCV and TEC. Haemato-biochemical changes were more severe in *B. bigemina* infected group in contrast to other groups and anaemia is becoming more severe as the disease progress due to extensive intravascular haemolysis.

Key words: Anaemia, *Babesia*, Cattle, Duplex PCR, Haemato-biochemical, *Theileria*

Bovine theileriosis and babesiosis are amongst the most abundant tick-borne diseases and exert their greatest impact in the tropical and subtropical regions imposing the major health impediments to efficient livestock production. The recovered animals also become persistent carriers and play an important role in disease epidemiology. These carrier animals play an important role in the transmission of the infection by ticks (Jonsson *et al.* 2008). Further, outbreaks of diseases occur when these animals with latent infections are being transported from endemic to non-endemic areas. The importance of these two diseases in India is due to severe economic losses and their effect on the immune status of the body. Marked changes revealed by Ganguly *et al.* (2017a) in haematological and biochemical parameters of *B. bigemina* infected crossbred cows may be beneficial in understanding the disease pathogenesis, undertaking appropriate corrective measures and effective treatment. Ganguly *et al.* (2017b) in their study revealed an increasing trend of prevalence of tick-borne haemoparasitic diseases and the presence of seasonal variation in occurrence of *T. annulata* and *B. bigemina* infection in Eastern Haryana, India. Nucleic acid based diagnosis like PCR has several advantages over frequently used conventional parasitological techniques (Bilgic *et al.* 2017). However conventional PCR can only detect a single target efficiently and is time consuming and expensive from epidemiological point of view. The goal of this study was to employ in-house standardized duplex PCR assay for simultaneous detection of *T. annulata* and *B. bigemina* (Maharana *et al.* 2018) and compare the effect of theileriosis, babesiosis and natural concurrent infections on the haemato-biochemical parameters in cattle.

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MATERIALS AND METHODS

Sample collection: Blood samples (327) were collected from jugular vein of suspected cattle brought to outpatient department (OPD) of veterinary unit, LUVAS, Karnal during July, 2017 to June, 2018 with the help of sterile syringe (5 ml) and reserved in a vial containing EDTA/ without EDTA and kept on ice. This whole blood was used for smear preparation, haematological examination and DNA isolation for duplex PCR assay. Serum was separated

from vials without EDTA after centrifugation at 5000 rpm for 15 min and stored at –20ºC till further use.

Grouping of animals: Based on microscopy (ME) and duplex PCR based assay, the animals naturally infected with haemoparasites were divided into three groups each having thirty animals, viz. Group I (*T. annulata* infected group; 30), Group II (*B. bigemina* infected group; 30), Group III (Animals which were having concurrent infection of *T. annulata* and *B. bigemina* considered as mixed infection group; 30). Group I and Group II were further subdivided into three subgroups based on severity of infection as latent, subclinical and clinical. Animals (10) free from any haemoparasitic infections (confirmed by PCR and ME) were considered as healthy control group (Group IV).

Duplex PCR: Following *in silico* analysis for candidate target genes representing each of the haemoparasites, an optimised duplex PCR assay was established using two sets of primers namely ssurRNA and cytb1 for genomic DNA amplification of *B. bigemina* and *T. annulata* encoding product size of 689 and 312 bp, respectively (Maharana *et al.* 2018). Briefly, the PCR reactions were set up into 25 µL volume containing 12.5 µL Top Taq® PCR Master Mix (QIAGEN, India) (Top Taq DNA polymerase 5 U/µL in PCR buffer, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 µL of each primer (10 pmol each of BBF/R and TAF/R) and 1 µL of the extracted DNA template and the total volume was made up to 25 µL using nuclease-free water. The PCR cycling conditions were set in automated thermal cycler (Applied Biosystem, USA) with the following programme: initial denaturation at 94ºC for 3 min, 32 cycles of denaturation at 94ºC for 30 sec, annealing at 50ºC for 1 min, extension at 72ºC for 1.2 min and the final extension at 72ºC for 10 min. For each reaction, 10 µL of PCR amplicon was analyzed by agarose gel electrophoresis in 2% agarose gel containing 10 µg/mL ethidium bromide in Tris-acetate-EDTA (TAE) buffer at 50 V for 1 h and visualized under UV light (Gel Doc^{TM} XR^{+}, BIORAD, USA).

Haematological analysis: Complete blood cell profile like haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total erythrocyte count (TEC), differential cell count, total leukocyte count (TLC) and thrombocytes count (THR) was analyzed using blood cell counter (MS4Se, MeletSchlosing lab).

Biochemical analysis: Biochemical parameters, viz. blood glucose, calcium, phosphorus, blood urea nitrogen, serum creatinine, total protein, albumin, globulin, aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TBIL) and direct bilirubin (DBIL) were estimated using fully automated Biochemistry analyzer (EM Destiny 180, Erba Diagnostic Mannheim GmbH).

Statistical analysis: One-way analysis of variance (ANOVA) was applied on various haematological and biochemical parameters to determine the variance in the animals of different groups using SPSS software (SPSS 20.0).

RESULTS AND DISCUSSION

Relative efficacy of duplex PCR and compound microscopy based detection: Total 327 blood samples were examined for infection with *T. annulata* and *B. bigemina* using microscopy and duplex PCR. Out of 327 samples, 107 (32.72%) and 17 (5.19%) samples were positive for *T. annulata* and *B. bigemina* respectively by microscopy (Table 1).

When the samples were screened by duplex PCR, 130 (39.75%) and 27 (8.25%) samples had single infections with *T. annulata* and *B. bigemina,* respectively. Duplex PCR was able to detect 36 (11%) cases of mixed infections compared to 9 (2.75%) by microscopy.

The assay could effectively diagnose *T. annulata* infection in 82.3% clinical, 9.2% subclinical, and 8.4% carrier cases while it effectively detected *B. bigemina* infection in 62.9% clinical, 14.8% subclinical (with clinical signs but negative by ME), and 22.2% latent cases (ME negative with no clinical signs). The assay could detect mixed infection in 25% clinical, 47.2% subclinical, and 27.7% latent cases (Table 2). These results defend the greater sensitivity of duplex PCR in detecting the subclinical and latent infections of both the haemoprotozoan. The molecular prevalence of *T. annulata* was reasonably higher than that of *B. bigemina* because of abundance of tick vector population, i.e. *Hyalomma anatolicum anatolicum* over *Boophilus microplus* in this geographical region. Earlier studies also suggested that *T. annulata* is the most prevalent and endemic hamoparasites in this region (Ganguly *et al.* 2017b).

The incidence of co-infection of both the haemoparasite was apparently less owing to different vectors responsible for their transmission (Sumbria *et al.* 2015). The results obtained using the field samples clearly indicate the prevalence of mixed infections in the field and substantiate the requirement for an assay that can simultaneously detect multiple pathogen species.

Effect of haemoptotozoan infections on haemato-biochemical profile of host animals: Group I, II and III showed significantly decreased levels of TEC, Hb, PCV and red blood cell indices (MCV, MCH and MCHC) (Table 3) as compared to healthy animals indicating microcytic hypochromic anaemia. In most anaemic conditions, alteration in average size of red cells are paralleled by

Table 1. Duplex PCR and blood smear examination on suspected cattle blood samples

Sample examination	Number of positive animals (%)			Total no. of animals
	T. annulata	*B. bigemina*	*T. annulata* + *B. bigemina*	
Microscopy	107 (32.72)	17 (5.19)	9 (2.75)	327
Duplex PCR assay	130 (39.75)	27 (8.25)	36 (11.0)	327

Figures in parenthesis indicate percentage.

Table 2. Correlation of infection of *Theileria annulata* and *Babesia bigemina* with sensitivity of Duplex PCR

Assay	*T. annulata* positive (130)			*B. bigemina* positive (27)			Mixed infection (36)		
	+/CS	-/CS	-/NCS	+/CS	-/CS	-/NCS	+/CS	-/CS	-/NCS
Duplex PCR positive	107 (82.3%)	12 (9.2%)	11 (8.4%)	17 (62.9%)	4 (14.8%)	6 (22.2%)	9 (25%)	17 (47.2%)	10 (27.7%)

+/CS, sample positive by light microscopy and animal exhibited clinical signs (clinical cases); -/CS, sample negative by light microscopy and animal exhibited clinical signs (subclinical cases); -/NCS, sample negative by light microscopy and animal did not exhibit clinical signs (latent cases).

similar changes in MCH and often the MCHC. With microcytic cells, the haemoglobin amount (MCH) is decreased and this is referred as a microcytic hypochromic anaemia. Similar findings have been reported by various scientists (Tuli *et al.* 2015, Ganguly *et al.* 2017a). The significant decrease in Hb, TEC, and PCV in infected groups may be due to intravascular haemolysis (Sharma *et al.* 2016) or down regulation of erythropoeitic activity of bone marrow (Pandey and Mishra 1987).

Marked thrombocytopenia and leucocytopenia was also observed in affected animals. The blood cellular changes revealed a significant (P<0.05) increase in mean per cent concentration of lymphocytes and decrease in mean per cent concentration of neutrophils in all the infected groups in comparison to healthy animals. Similar results were reported by Tuli *et al.* (2015).

Splenomegaly, which is associated with many of the tick-borne diseases, causes increased platelet sequestration and destruction by splenic macrophages (Hildebrandt 1981). Relative increase in number of lymphocytes in case of theileriosis reflects compensatory mechanism as target cells (lymphocytes) undergo retrogressive metamorphosis in

Table 3. Mean values[#] of haemato-biochemical parameters of crossbred cattle infected with *Theileria annulata, Babesia bigemina* and mixed infection

Parameter	Group I	Group II	Group III	Group IV	Range of parameters in healthy group
	Cattle infected with *Theileria annulata* (30)	Cattle infected with *Babesia bigemina* (30)	Cattle infected with mixed infection (30)	Healthy cattle (10)	
Hb (gm/dL)	6.33±0.26^{a}	5.02±0.27^{b}	5.31±0.31^{b}	12.77±0.11^{c}	12.2–13.5
PCV (%)	21.38±0.85^{a}	18.07±0.98^{a}	20.75±1.15^{a}	38.70±0.66^{b}	37–41
TEC (×10^{6}/µL)	4.69±0.20^{ap}	3.99±0.22^{aq}	4.10±0.203^{aq}	6.90±0.108^{b}	6.25–7.23
TLC (×10^{3}/µL)	5.71±0.59^{a}	7.6±0.99^{a}	6.06±1.00^{a}	9.36±2.15^{b}	8.0–10.6
Lymphocyte (%)	62.68±1.862^{p}	62.14±2.62^{p}	58.02±2.34^{p}	50.60±1.75^{q}	42–60
Neutrophil (%)	30.77±1.75^{p}	31.96±2.46^{p}	36.16±2.32^{p}	45.90±1.80^{q}	40–56
MCV (fl)	46.23±1.38^{ap}	45.99±1.24^{ap}	50.88±1.49^{aq}	56.10±1.139^{b}	49.85–60.51
MCH (pg)	14.35±0.88^{ap}	12.65±0.35^{aq}	12.90±0.44^{aq}	18.52±0.34^{b}	16.87–19.51
MCHC (%)	29.62±0.54^{a}	27.96±0.84^{a}	25.86±0.93^{a}	33.06±0.504^{b}	31.21–36.76
THR (× 10^{3}/µL)	283.6±42.02^{a}	112.36±11.77^{b}	147.97±17.06^{b}	333.20±9.965^{c}	298–360
Ca (mg/dL)	7.9±0.13^{a}	7.71±0.22^{a}	7.82±0.29^{a}	9.98±0.26^{b}	8.5–11.5
P (mg/dL)	4.3±0.19^{a}	4.12±0.24^{a}	5.14±0.32^{a}	4.78±0.13^{a}	4.2–5.4
ALT (U/L)	24.3±2.14^{a}	23.3±1.82^{a}	31.73±2.73^{a}	28.7±2.31^{a}	21–42
AST (U/L)	141.6±13.06^{a}	145.7±13.45^{a}	182.71±16.31^{a}	76±2.81^{b}	67–87
Glucose (mg/dL)	52.6±2.01^{p}	56.07±1.34^{p}	53.2±2.38^{p}	65.6±1.82^{q}	55–69
TSP (g/dL)	5.8±0.129^{a}	5.27±0.11^{a}	5.37±0.19^{a}	7.03±0.164^{b}	6.2–7.8
Albumin (g/dL)	2.6±0.10^{p}	2.55±0.087^{p}	2.57±0.10^{p}	3.2±0.06^{q}	3–3.5
Globulin (g/dL)	3.3±0.18^{p}	2.719±0.10^{q}	2.80±0.17^{q}	3.84±0.19^{p}	2.8–4.7
BUN (mg/dL)	19.4±1.26^{p}	22.6±1.43^{p}	20.33±1.62^{p}	20.63±0.63^{p}	18.2–23.5
Cr (mg/dL)	1.1±0.043^{p}	1.12±0.15^{p}	1.28±0.10^{p}	1.26±0.77^{p}	0.9–1.5
TBIL (mg/dL)	0.3±0.06^{p}	0.70±0.07^{q}	0.91±0.19^{r}	0.68±0.108^{q}	0.2–1.2
DBIL (mg/dL)	0.2±0.03^{p}	0.42±.0.045^{p}	0.46±0.037^{p}	0.3±0.03^{p}	0.1–*0*.5
IBIL (mg/dL)	0.2±0.036^{p}	0.33±0.078^{p}	0.82±0.077^{q}	0.38±0.072^{p}	0.1–*0*.7
A:G	0.9±0.074^{p}	1.02±0.09^{p}	1.05±0.099^{p}	0.85±0.06^{p}	0.66–1.21

[#]Mean±SEM; [abc]Infected and healthy cattle differ significantly at P<0.001; [pqr]significant at P<0.05. Means with different superscripts in same row differ significantly. Hb, Haemoglobin; PCV, packed cell volume; TEC, total erythrocyte count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TLC, total leukocyte count; THR, thrombocytes; Ca, calcium; P, phosphorus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TSP, total serum protein; BUN, blood urea nitrogen; Cr, creatinine; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; A:G, albumin globulin ratio.

order to divide synchronously with the haemoparasites in response to their invasion. Marked anaemia and leucopenia were commonly observed in bovine theileriosis (Ceci *et al.* 1997). Anaemia could be attributed to toxin metabolites of *Theileria* which have harmful effects on bone marrow by interfering with the process of erythropoiesis. Additionally, piroplasm causes severe destruction of RBCs resulting in anaemia. Persistent loss of blood by ticks could play a significant role as well. Apart from that, TNF-α has been considered to induce anaemia by suppressing haematopoietic progenitors (Boulter and Hall 2000). The insignificant changes in total leucocyte count in *B. bigemina* infected group may be due to the destruction of red blood cells by the protozoan stimulating phagocytic cells such as lymphocytes and monocytes to clean up the toxic remnants of ruptured red blood cell (Mohamed 2017).

Theileria annulata and *B. bigemina* infected groups were further divided into three subgroups (latent, subclinical and clinical) on the basis of severity of infection. In case of *Theileria annulata* infected group, out of 30 animals, 13 cases of latent, 7 cases of subclinical and 10 cases of clinical infection were observed while the numbers were 4, 10 and 16 respectively in case of *B. bigemina* infected group. In both the cases, significant decline was observed in mean values of haemoglobin (P<0.001), PCV (P<0.001) and TEC (P<0.05) as the disease progress from latent infection to clinical infection (Tables 4 and 5). These differences may be due to excessive intravascular haemolysis as the severity of infection increased. In other parameters, no significant difference was noticed.

Leucocytopenia was observed in subclinical and clinical cases of *B. bigemina* infected animals and in all the stages of *T. annulata* infected animals whereas leukocytosis was only noticed in latent infection of *B. bigemina*. Significant decrease (P<0.05) in the mean level of thrombocytes was observed in clinical and subclinical theileriosis cases. Significant decrease (P<0.001) in thrombocytes count was noticed in latent and other two stages of babesiosis cases. No significant difference was monitored in other haemogram parameters among the three stages of both the infections. It is evident from the above findings that with the severity of disease, there is more and more destruction of RBCs leading to decline in concerned parameters. Marked anaemia in clinical babesiosis cases has been recorded in previous studies (Aulakh *et al.* 2005, Ganguly *et al.* 2017a). Serum biochemistry of infected cows (Group I, II and III) showed significantly higher values of AST and low levels of blood glucose, calcium, total protein, albumin as compared to healthy counterparts (Table 3) revealing hypocalcemia, hypoglycemia, hypoproteinemia and hypoalbuminemia in the infected groups.

The significant rise in AST activity may be due to the harmful effect of toxic metabolites of *Theileria* and *Babesia* spp. on liver cells (Ganguly *et al.* 2017a, Pandey *et al.* 2017). Decrease in serum calcium level may be attributed to hypoalbuminaemia. It is important to mention that a high proportion of calcium is bound to albumin. However, it is the unbound calcium that is the most important physiologically. Therefore when albumin is low, the total calcium level may be misleading (Hamilton and Bickle 2006).

Table 4. Haemato-biochemical indices (Mean±SEM) of latent, subclinical and clinical cases of *Theileria annulata* infected crossbred cattle

Parameter	Infected cattle (30)		
	Latent (13)	Subclinical (7)	Clinical (10)
Hb (gm/dL)	7.81±0.177^{a}	6.79±0.10^{b}	4.71±0.40^{c}
PCV (%)	25.71±0.76^{a}	23.06±0.49^{b}	16.18±1.24^{c}
TEC (×10^{6}/µL)	5.75±0.24^{ap}	5.01±0.191^{aq}	3.558±0.238^{b}
TLC (×10^{3}/µL)	6.00±0.88^{p}	5.56±0.877^{p}	5.55±0.87^{p}
Lymphocyte (%)	60.23±2.83^{p}	65.6±2.34^{p}	65.62±2.24^{p}
Neutrophil (%)	33.6±2.68^{p}	27.75±1.86^{p}	27.73±2.33^{p}
MCV (fl)	45.0±2.21^{p}	47.25±2.52^{p}	45.79±2.61^{p}
MCH (pg)	13.79±0.52^{p}	13.72±0.52^{p}	15.56±2.57^{p}
MCHC (%)	30.69±0.94^{p}	29.5±0.80^{p}	28.96±1.11^{p}
THR (×10^{3}/µL)	322.29±127.38^{p}	296.23±58.99^{p}	240.1±56.03^{p}
Ca (mg/dL)	8.4±0.19^{p}	7.38±0.20^{p}	7.6±0.12^{p}
P (mg/dL)	4.0±0.40^{p}	4.5±0.38^{p}	4.2±0.118^{p}
ALT (U/L)	26.7±5.43^{p}	20.3±1.17^{p}	27.8±4.92^{p}
AST(U/L)	161.2±34.68^{p}	164.0±8.51^{p}	168.8±26.59^{p}
Glucose (mg/dL)	56.9±2.0^{p}	50.8±3.45^{p}	51.9±3.82^{p}
TSP (g/dL)	5.8±0.30^{p}	5.9±0.20^{p}	5.7±0.21^{p}
Albumin (g/dL)	2.6±0.19^{p}	2.4±0.15^{p}	2.7±0.22^{p}
Globulin (g/dL)	3.2±0.38^{p}	3.5±0.29^{p}	3.0±0.28^{p}
BUN (mg/dL)	18.8±2.32^{p}	14.1±1.03^{p}	17.8±3.10^{p}
Cr (mg/dL)	1.1±0.10^{p}	1.0±0.05^{p}	1.2±0.08^{p}
TBIL (mg/dL)	0.4±0.133^{p}	0.3±0.02^{p}	0.5±0.12^{p}
DBIL (mg/dL)	0.2±0.052^{p}	0.2±0.03^{p}	0.3±0.08^{p}
IBIL (mg/dL)	0.2±0.10^{p}	0.1±0.028^{p}	0.2±0.073^{p}
A:G	0.9±0.13^{p}	0.8±0.114^{p}	1.0±0.138^{p}

Values with different superscripts in same row differ significantly. (^{abc}P<0.001; ^{pqr}P<0.05). Hb, Haemoglobin; PCV, packed cell volume; TEC, total erythrocyte count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TLC, total leukocyte count; THR, thrombocytes; Ca, calcium; P, phosphorus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TSP, total serum protein; BUN, blood urea nitrogen; Cr, creatinine; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; A:G, albumin globulin ratio.

Hyperbilirubinemia was observed in mixed infection group. Omer *et al.* (2002) reported similar findings of higher level of TBIL in animals infected with *T. annulata* resulting from the destruction of parasitized erythrocytes by reticulo-endothelial system. Activity of AST was also increased in all the infected groups as compared to control. AST is the marker for soft tissue damage and liver. Other parameters remained constant in all groups. Decrease in blood glucose level may be due to high to moderated fever along with anorexia. Exhaustion of energy resources further leads to weakness. No significant difference in the biochemical parameters was observed among latent, subclinical and clinical cases of *T. annulata* (Table 4) though there was significant difference between the biochemical parameters

Table 5. Haemato-biochemical indices of latent, subclinical and clinical cases of *Babesia bigemina* infected crossbred cattle

Parameter	Infected cattle (30)		
	Latent (4)	Subclinical (10)	Clinical (16)
Hb (gm/dL)	7.38 ± 0.47^{a}	5.82 ± 0.14^{b}	3.94 ± 0.24^{c}
PCV (%)	25.95 ± 3.17^{a}	20.06 ± 0.97^{b}	14.86 ± 0.89^{c}
TEC($\times10^{6}$/µL)	5.89 ± 0.47^{p}	4.38 ± 0.225^{q}	3.29 ± 0.22^{r}
TLC ($\times10^{3}$/µL)	12.24 ± 1.19^{a}	4.41 ± 3.25^{b}	4.5 ± 0.49^{b}
Lymphocyte (%)	63.03 ± 4.58^{p}	62.94 ± 5.9^{p}	61.07 ± 2.69^{p}
Neutrophil (%)	31.25 ± 4.76^{p}	30.86 ± 5.45^{p}	33.19 ± 2.43^{p}
MCV (fl)	44.08 ± 3.56^{p}	46.75 ± 3.0^{p}	45.99 ± 1.22^{p}
MCH (pg)	12.53 ± 0.30^{p}	13.48 ± 0.59^{p}	12.16 ± 0.52^{p}
MCHC (%)	29.10 ± 2.21^{p}	29.61 ± 1.67^{p}	26.65 ± 1.02^{p}
THR ($\times10^{3}$/µL)	185.75 ± 53.19^{a}	106.5 ± 16.69^{b}	91.44 ± 12.47^{b}
Ca (mg/dL)	7.8 ± 0.53^{p}	8.1 ± 0.33^{p}	7.5 ± 0.34^{p}
P (mg/dL)	4.93 ± 1.05^{p}	4.1 ± 0.31^{p}	3.92 ± 0.31^{p}
ALT (U/L)	29.92 ± 4.29^{p}	24.6 ± 2.66^{p}	23.98 ± 2.85^{p}
AST (U/L)	87 ± 11.31^{p}	120 ± 11.23^{q}	177.10 ± 21.13^{r}
Glucose (mg/dL)	55.95 ± 5.10^{p}	55.16 ± 1.65^{p}	56.67 ± 1.87^{p}
TSP (g/dL)	$5.58\pm.12^{p}$	5.2 ± 0.25^{p}	5.32 ± 0.142^{p}
Albumin (g/dL)	2.84 ± 0.23^{p}	2.6 ± 0.208^{p}	2.45 ± 0.082^{p}
Globulin (g/dL)	2.73 ± 0.22^{p}	2.6 ± 0.19^{p}	2.86 ± 0.11^{p}
BUN (mg/dL)	16.71 ± 4.36^{p}	20.3 ± 2.60^{p}	31.6 ± 3.09^{q}
Cr (mg/dL)	1.3 ± 0.118^{p}	1.1 ± 0.12^{p}	1.37 ± 0.09^{p}
TBIL (mg/dL)	0.46 ± 0.102^{p}	0.55 ± 0.1^{p}	0.87 ± 0.108^{q}
DBIL (mg/dL)	0.28 ± 0.068^{p}	0.3 ± 0.005^{p}	0.54 ± 0.06^{q}
IBIL (mg/dL)	0.18 ± 0.093^{p}	0.25 ± 0.097^{p}	0.33 ± 0.078^{q}
A:G	1.08 ± 0.18^{p}	1.1 ± 0.14^{p}	0.87 ± 0.04^{p}

Values with different superscripts in same row differ significantly. ($^{abc}P<0.001$; $^{pqr}P<0.05$). Hb, Haemoglobin; PCV, packed cell volume; TEC, total erythrocyte count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TLC, total leukocyte count; THR, thrombocytes; Ca, calcium; P, phosphorus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TSP, total serum protein; BUN, blood urea nitrogen; Cr, creatinine; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; A:G, albumin globulin ratio.

of *T. annulata* infected and control group (Table 3).

When we compared the biochemical parameters of subgroups of *Babesia bigemina* infected group (Table 5), significant difference ($P<0.05$) was observed among calcium level, TBIL, DBIL, IBIL and AST activity. Hypocalcemia can trigger a cascade of negative events that ultimately reduce dry matter intake, increase metabolic diseases and secondary infections such as haemoprotozoan infection, GI disorders, gynaecological disorders, parasitic and other infections (Ganguly *et al*. 2015). On the progress of the severity of disease, there is increase in the level of BUN as well as total, indirect and direct bilirubin. Significant increase in the activity of AST was also observed which indicates the harmful effect of toxic metabolites of *Babesia bigemina* on the organs like soft tissue, liver, kidney. These results are supported by the findings of Hussein *et al.* (2007) and Mohamed (2017). Significant increase of bilirubin levels in affected animals may be due to haemolytic crisis of babesiosis (Panday and Misra 1987) and hepatic injury (Yeruham *et al.* 2003). The concentration of BUN in diseased animals may be due to increased turnover of proteins. No significant difference was recorded in creatinine levels in animals found positive for *T. annulata, B. bigemina* and mixed infection in comparison to control (Group IV). Except AST, no significant difference was observed in other biochemical parameters among latent, subclinical and clinical groups of *B. bigemina* infected animals (Tables). The increase in enzyme activity may be attributed to anaemia that lead to hypoxic and toxic liver damages (Allen *et al.* 1981)

It can be concluded that haemato-biochemical changes were more severe in *B. bigemina* infected group as compared to *T. annulata* and mixed infection group. Anaemia becomes more severe as the disease progress due to extensive intravascular haemolysis. Along with molecular diagnosis, haemato-biochemical observation supported the pathogenicity of the single and concurrent infection. Latent carrier and subclinical infections diagnosed by molecular means were also responsible for inducing pathogenicity.

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