

## Short Communication

### Clinicopatho-Biochemical Alterations Associated with Subclinical Babesiosis in Dairy Animals

Amrita Sharma, \*Lachhman Das Singla, Ashuma, Balvinder Kaur Batth, Paramjit Kaur

Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

(Received 13 Apr 2014; accepted 16 Sep 2014)

#### Abstract

**Background:** Present investigation is based on the clinicopatho-biochemical alteration related to natural tick borne bovine babesiosis in Punjab state.

**Methods:** Blood samples from jugular vein of 542 bovines (cattle 466 and buffaloes 76) having history of tick infestation, fever, hemoglobinuria or anemia were collected and tested for *Babesia bigemina* by blood smear examination and PCR targeting 18S rRNA gene to distinguish clinically and subclinically infected groups. Further the hemato-biochemical parameters were correlated with the status of infection.

**Results:** Overall, of the 542 samples tested 16.42 % were positive by PCR while only 1.66 % by blood smear examination. The trend of molecular prevalence was found to decrease from north-eastern towards western Punjab. Analysis of the hematobiochemical alterations showed significant decrease in the levels of RBC, Hb, PCV, and MCV with significant increase in TBIL, MCH and MCHC levels.

**Conclusion:** As the transmission of *B. bigemina* is transovarian, presence of even few infected *Rhipicephalus (Boophilus) microplus* ticks on a subclinically infection can be the nidus of infection for whole herd, causing severe economic losses, at the same time significantly affecting the physiology of carrier animal.

**Keywords:** Babesiosis, Dairy animals, Haemato-biochemical, Prevalence, PCR

#### Introduction

Bovine babesiosis, caused by *Babesia bigemina*, is an important tick-borne disease in the tropical and subtropical counties, transmitted by *Rhipicephalus (Boophilus) microplus*. India suffers losses of about 57.2 million US dollars annually due to babesiosis and anaplasmosis in livestock (McLeod and Kristjanson 1999). Microscopic techniques like Giemsa stained blood smear is the most appropriate for the diagnosis of acute babesiosis but the low sensitivity of these method do not permit to identify the carrier animals (Juyal et al. 1994, Almeria et al. 2001, Singh et al. 2003). Serological tests, though employed for epidemiological survey of babesiosis, lack discrimination between previous and current exposure to infection (Passos et al. 1998). Molecular tests, such as PCR assays, are highly specific and sensitive method

over the existing diagnostic techniques for the diagnosis of subclinical infections (Figueroa et al. 1992). The molecular tests can depict the status of active infect in exposed animals and further their correlation with haemato-biochemical parameters will provide an insight into the pathogenicity of subclinical infection primarily diagnosed by PCR assay.

The present study was therefore undertaken to diagnose subclinical cases of babesiosis by PCR based molecular diagnosis in relation to the haemato-biochemical parameters to study the pathogenicity induced by latent infection of *B. bigemina*.

#### Materials and Methods

##### Sampling frame

The province of Punjab covers a total

area of 50,362 square kilometers between 29°30'N to 32°32'N latitude and 73°55'E to 76°50'E longitudes. The study area was divided into three regions: Western Punjab (Western and Western Plain Zone), Central Punjab (Central Plain Zone) and North-eastern Punjab (Submountain and Undulating Zone) (Fig. 1). To study the status of molecular prevalence of the babesiosis caused by *B. bigemina*, the expected prevalence to be 50% with confidence limits of 95% and a desired absolute precision of 5% to collect maximum number of samples was considered. The number of samples thus calculated was adjusted for finite population (Thrusfield 2005) and correlated with 542 samples (cattle 466 and buffaloes 76) collected from month of May to October, 2011 from jugular vein of dairy animals having history of tick infestation, fever, hemoglobinuria or anemia from Punjab state (India) also including the samples from the outbreaks of *B. bigemina* in central and north-eastern regions. About 5ml of blood was collected aseptically from the jugular vein of each animal in EDTA coated vials for DNA isolation and serum separation, respectively. The extracted DNA and collected sera were stored at -20 °C for further analysis.

#### Clinical and subclinical diagnosis

Animals diagnosed positive by Giemsa stained thin blood smears technique were considered clinically positive cases. For sub-clinical diagnosis, DNA was extracted using DNA isolation Kit (HiPura Blood Genomic DNA Miniprep Purification Spin Kit) as per the protocol of the manufacturer. The Bg3/Bg4 set of oligonucleotide primer was used to amplify 18S ribosomal RNA gene (Ellis et al. 1992). The nucleotide sequence of the primer was Bg3 5' TAGTTGTATTTTCAGCCTCGCG 3' and Bg4 -5' AACATCCAA GCAGCTAHTTTAG 3'. The PCR reaction mixture (25µl) mixture constituted of 12.5µl of KAPA 2G™ Fast Hot Start Ready Mix

(2X containing KAPA2G fast hot start DNA polymerase, KAPA 2G fast hot start PCR buffer, 0.2 mM dNTP each, 1.5 mM MgCl<sub>2</sub>), 3 µl of 25 mM MgCl<sub>2</sub>, 1.5 µl of 10 pmol Bg3/Bg4 primers, 1.5 µl DEPC-treated water and 5 µl of DNA template. The reaction was carried on in automated Thermocycler (Eppendorf, Master Cycler Personal) on the following programme: initial denaturation at 95 °C (5min), 30 cycles of denaturation at 95 °C (30 sec), annealing at 57 °C (1min), and extension at 72 °C (1.5 min) with final extension at 72 °C for 10 min. The amplified PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV Transilluminator for detection of 689 bp amplified product.

#### Haemato-biochemical analysis

The hematology was done on ADVIA 2120 Hematology System (Siemens Health Care Diagnostic Inc. Deerfield, IL, USA) and serum biochemical profile was drawn on VTROS DT 6011 System Chemistry using Ortho-Clinical Diagnostics kit (Johnson and Johnson Company).

#### Nucleotide sequence analysis

The 689 bp product obtained by the Bg3/Bg4 primers specific for *B. bigemina* PCR were custom sequenced from Xcelris Genomics, Ahmedabad, India. The nucleotide sequences were subjected to BLASTn analysis (Altschul et al. 1990) for determining the similarity with the sequences present in the nucleotide database.

#### Statistical analysis

Chi-square test was applied to evaluate association of disease prevalence with various districts under study. One-way Analysis of Variance (ANOVA) was applied to various haematological and biochemical parameters to determine the variance in these parameters using SPSS software. Agreement between the results of the two techniques was analyzed through Cohen's Kappa coefficient.

## Results

### Diagnosis of infection

Out of total 542 blood samples (466 cattle and 76 buffaloes), PCR based (Fig. 2) diagnosis revealed 89 animals (16.42%, 95% CI= 13.73–19.1) positive for *Babesia* that included 83 cattle (17.81%, 95% CI= 14.81–20.81) and 6 buffaloes (7.89%, 95% CI= 2.66–13.12). The relative prevalence of *B. bigemina* by PCR amplifying 689 bp fragment for 18S rRNA gene showed highest prevalence of the disease in North-eastern region (64.28%, 95% CI= 51.79–76.78) (Fig. 1) and lowest in western region (3.89%, 95% CI= 1.45–6.32) of Punjab. Overall Overall percentage of animals positive for *B. bigemina* by Giemsa-stained thin blood smears was 1.66 % (9/542), the higher in cattle 1.73 % (8/463) than in buffaloes 1.31 % (1/76). Chi-square test showed significant difference in the prevalence of the infection as revealed by blood smear examination and PCR. Kappa contingency test indicated a slight agreement between blood smear examination and PCR assay with latter being highly sensitive for the detection of latent infections.

### Nucleotide sequence analysis

The 689bp sequence showed homology (99%) with *B. bigemina* isolate BR02 18S ribosomal RNA gene (GenBank No. FJ 426361.1).

### Haemato-biochemical alteration

#### Anaemia

Haematological parameters (Fig. 2) showed significant ( $P < 0.05$ ) decrease in the TEC, Hb, PCV and MCV, while significant increase ( $P < 0.05$ ) in MCH and MCHC in clinically and subclinically infected animals as compared to non-infected healthy control group (Fig. 3).

### Liver function biochemical alterations

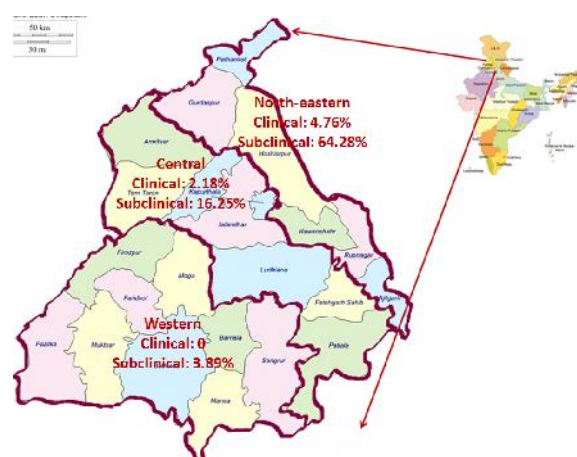
A significant increase ( $P < 0.05$ ) was seen in ALKP and AST of clinically infected group while the increase as non-significant in subclinically infected animals as compared to non-infected control group. The increase in ALT levels was non-significant ( $P < 0.05$ ) in both the infected groups (Fig. 4) when compared with the non-infected healthy controls.

### Kidney function biochemical alterations

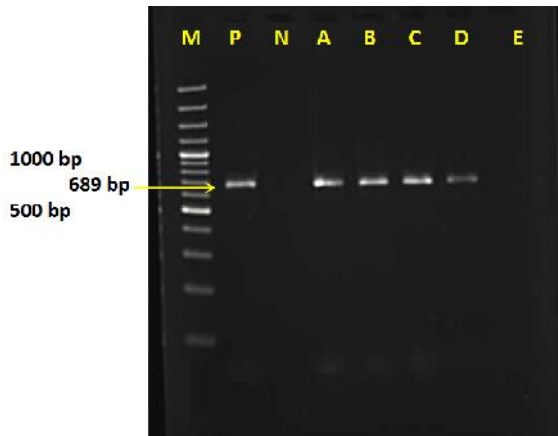
There was no significant difference in the level of creatinine and BUN in the three groups under study, however the level of total bilirubin had increased significantly in the infected groups as compared to non-infected group ( $P < 0.05$ ) (Fig. 5).

### Blood cellular and biochemical response

Significant response in terms of decrease in WBC, TP, GLO and Plt was seen only in clinically infected animals ( $P < 0.05$ ), while the alterations in albumin level was non-significant in both the infected groups as compared to non-infected control group (Fig. 6).

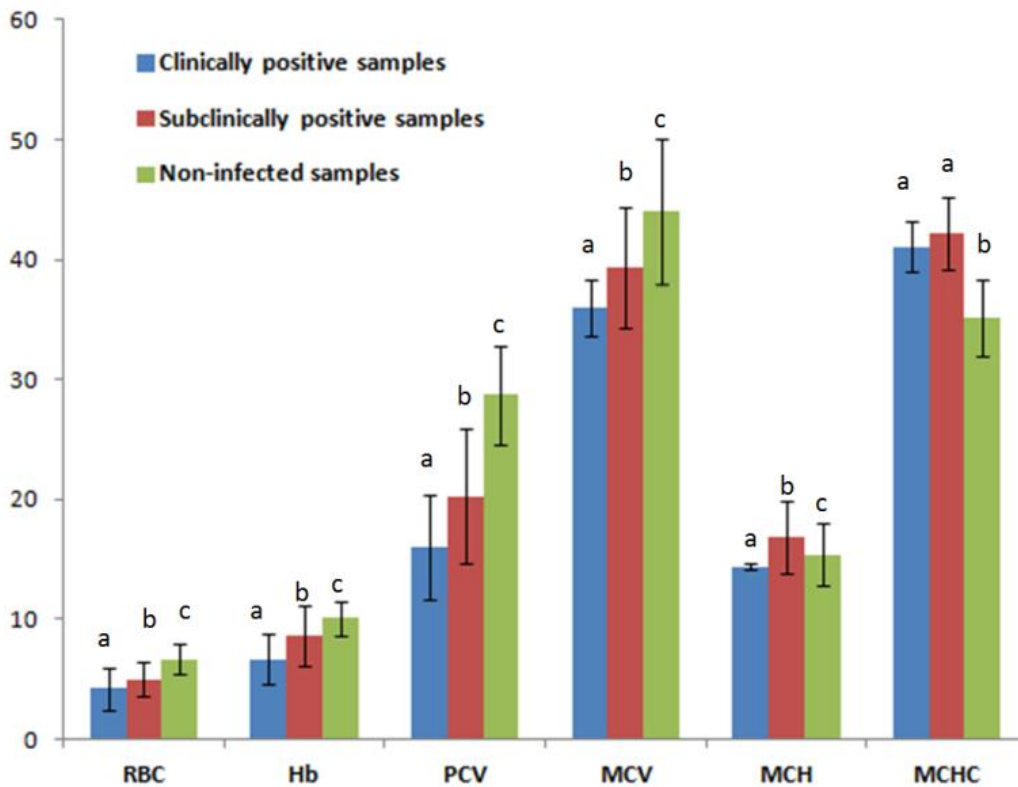


**Fig. 1.** Geographic distribution clinical and subclinical babesiosis of bovine babesiosis in Punjab

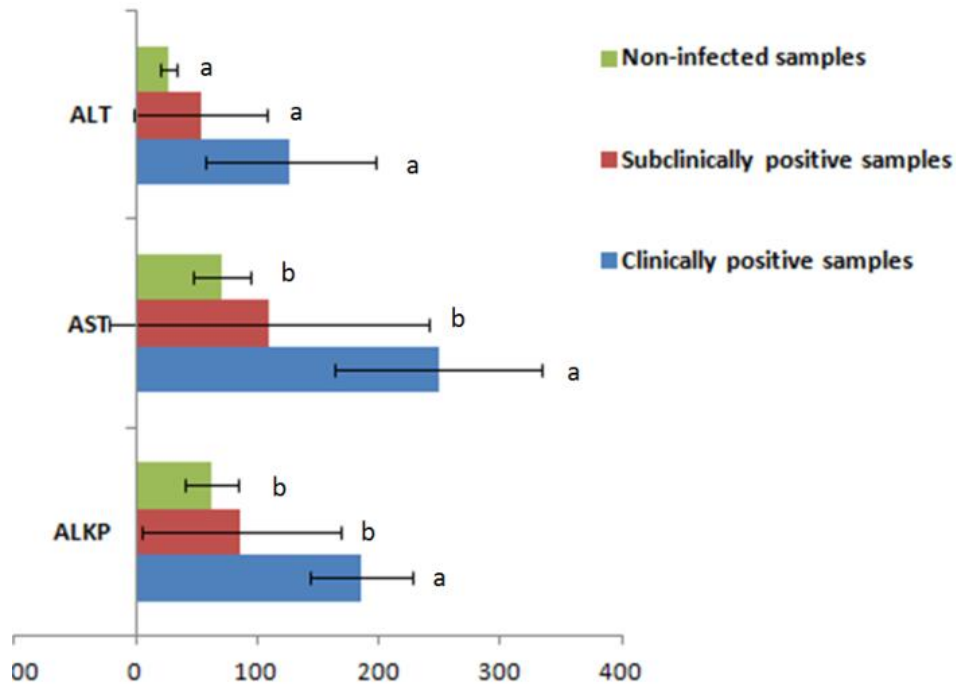


Lane M, molecular size marker 100 bp plus, Lane P positive control and N negative controls, Lane A–D showing amplified *B. bigemina* genomic DNA from the blood of animals positive for infection, Lane E showing no amplification of *B. bigemina* genomic DNA from the blood of animal negative for infection.

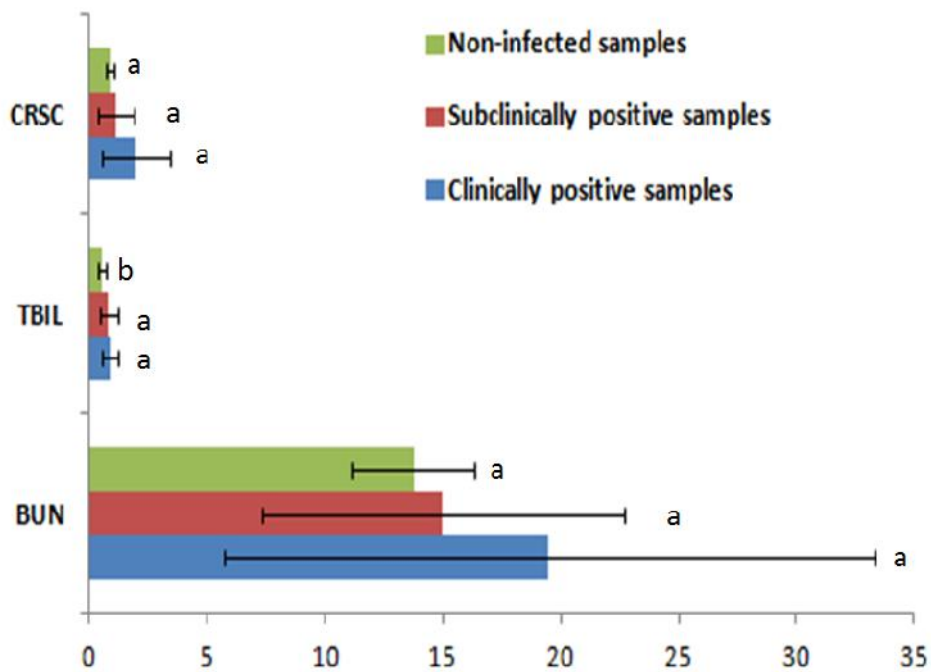
**Fig. 2.** Agarose gel (1.5%) electrophoresis showing amplified DNA (689bp) from *Babesia bigemina* targeting SSU rRNA gene using primer Bg3/Bg4



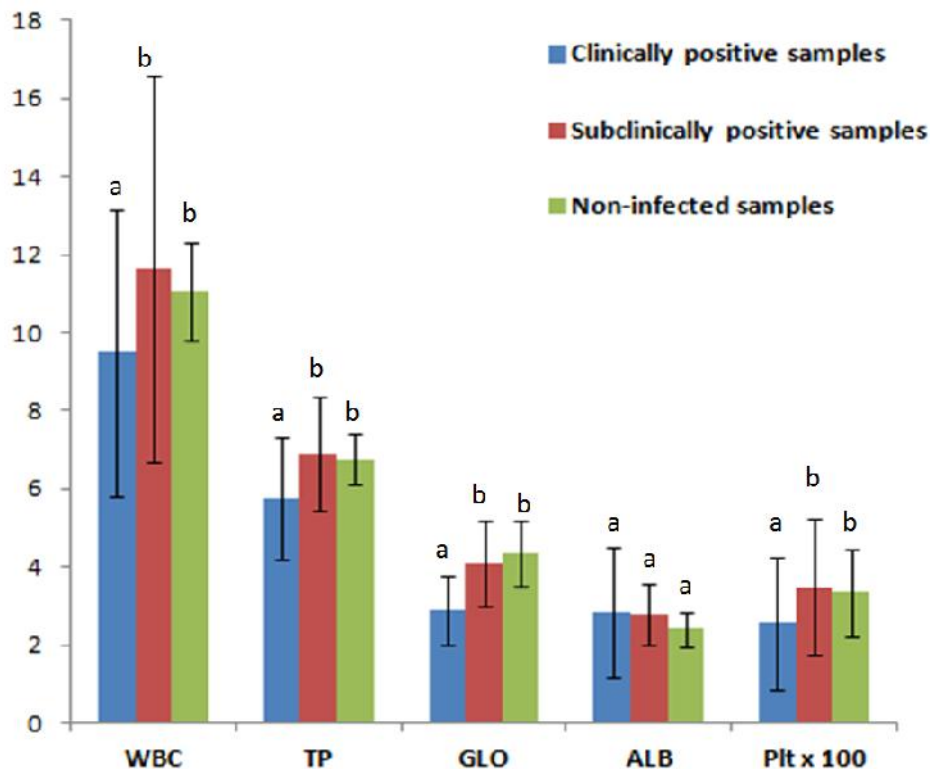
**Fig. 3.** Hematological alterations indication anemia in clinically and subclinically infected animals (RBC (Red blood cells):  $10^6$ cells/  $\mu$ l, HB (Hemoglobin level): g/dL, PCV (Packed cell volume):%, MCV (Mean corpuscular volume: fL, MCH (Mean corpuscular hemoglobin): pg, MCHC (Mean corpuscular hemoglobin concentration): g/dl). Bars with different alphabets a, b and c differ significantly for the parameter



**Fig. 4.** Liver function biochemical alterations in clinically and subclinically infected animals (ALKP (Alkaline phosphatase), AST (Aspartate aminotransferase), ALT (Alanine aminotransferase))



**Fig. 5.** Kidney function biochemical alterations in clinically and subclinically infected animals (CRSC (Cretinine), TBIL (Total bilirubin), BUN (blood urea nitrogen))



**Fig. 6.** Blood cellular and biochemical response in clinically and subclinically infected animals (WBC (White blood cells), TP (Total Protein), GLO (Globulin), ALB (Albumin), Plt (Platelets))

## Discussion

In the clinically positive (9) cases the marked clinical symptoms of high fever (39.4–40.5°C), history of hemoglobinuria, jaundice, icterus, tachycardia, weight loss and decrease in milk yield were recorded. The blood smear of these animals revealed typical pyriform bodies of *B. bigemina* at an acute angle inside the erythrocytes. Apart from the typical forms other pleomorphic forms *B. bigemina* were also observed. Previously, parasitological prevalence of babesiosis due to *B. bigemina* in cattle is reported to be 5.94% in Punjab (Aulakh et al. 2005). However, the status of subclinical infection is essential to be drawn as this kind of infection can be the source of infection to other animals of the herd as well as may flair in the condition of stress. Thus, it has been ob-

served that the molecular approaches based on nucleic acids such as polymerase chain reaction (PCR) assays offer greater sensitivity and specificity over the existing diagnostic tests (Fahrimal et al. 1992) which proved to be true in the present study also.

The trend of geographic distribution of babesiosis can be corroborated with the availability of favorable conditions of high humidity and soil moisture content (Ghai et al. 2008) and higher population of tick vector, *R. microplus* (Singh et al. 2000), in North-eastern parts are Punjab as it covers the Sub mountain and Undulating zones. Ticks were observed on 78.6% of the affected cattle and 9.4% of the affected buffaloes. Population of the ticks was mainly constituted by *R. (Boophilus) microplus* followed by *Hyalomma an-*

*natolicum anatolicum*, correlating the fact that incidence of *B. bigemina* is a tick borne pathogenesis. Above all the major fraction of sampling was done from cattle and they primarily considered susceptible to the infection (Vohara et al. 2012).

Findings of hematobiochemical profiling indicate that the subclinical infection of babesiosis in bovines is also responsible for causing anemia. The anemia was normo to hyperchromic and microcytic type. Marked anemia has been documented in clinical cases of bovine babesiosis in previous studies (Sharma et al. 2000, Aulakh et al. 2005) also. A number of factors contribute to anemia in babesiosis including lyses of erythrocytes by emerging parasites (Dwivedi et al. 1976), indiscriminate phagocytosis of infected and non-infected erythrocytes by activated macrophages system (Ruprah 1985), suppression of erythropoietic activity of bone marrow (Pandey and Misra 1987) and antigen antibody mechanism (Ferris et al. 1967). Non-significant difference was seen in glucose level among the three groups, when compared with the normal range (42–75 g/dL) (Latimer et al. 2005).

The apparent increase in the levels of AST, ALT, ALKP, BUN and creatinine signifies harmful effect of toxic metabolites of *Babesia* sp. on liver cells lead to impairment and alterations of the liver and kidney enzymes. Hussein et al. 2007 reported the significant increase in aspartate aminotransferase, alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT) in babesiosis. A significant increase in total bilirubin in subclinically infected group hemolytic crisis of babesiosis (Panday and Misra 1987) and hepatic damage (Yeruham et al. 2003) was seen. Overall, the degenerative changes in the internal organs as indicated by biochemical response may be due to anemic hypoxia. Infiltration of circulating immune complexes may further complicate the problem (Aulakh et al. 2005).

## Conclusion

The comparison of prevalence of bovine babesiosis by PCR and Giemsa stained thin blood smear in dairy cattle of Punjab, revealed highest prevalence in north-eastern region of Punjab by both the techniques. As the transmission of *B. bigemina* is transovarian, the presence of rather few infected ticks on even a subclinically infected animal may acts as nidus of infection for the whole herd, posing severe economic losses and pathognomic effects on the animal as indicated by altered vital parameters contemplated though significant decrease in the level of RBC, Hb, PCV, and MCV with significant increase in TBIL, MCH and MCHC levels.

## Acknowledgements

Thanks are due to the Dean, College of Veterinary Science and Director of Research, Guru Angad Dev Veterinary and Animal Sciences University for providing financial support to carry out the research work under RKVY project. The authors declare that there is no conflict of interests.

## References

- Almeria S, Castella J, Ferrer D, Ortuno A, Estrada-Pena A, Gutierrez JF (2001) Bovine piroplasms in Minorca (Balearic Islands, Spain): A comparison of PCR-based and light microscopy detection. *Vet Parasitol.* 99: 249–259.
- Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol.* 215: 403–410.
- Aulakh GS, Singla LD, Kaur P, Alka (2005) Bovine babesiosis due to *Babesia bigemina*: haematobiochemical and therapeutic studies. *Ind J Anim Sci.* 75: 617–622.

- Dwivedi SK, Sharma SP, Gautam OP (1976) Babesiosis: clinical cases in exotic and cross bred cattle. *Ind Vet J.* 55: 469–472.
- Ellis J, Hefford C, Baverstock PR, Dalrymple BP, Johnson AM (1992) Ribosomal DNA sequence comparison of *Babesia* and *Theileria*. *Mol Biochem Parasitol.* 54: 87–95.
- Fahrimal Y, Goff WL, Jasmer DP (1992) Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. *J Clin Microbiol.* 30: 1374–1379.
- Ferris DH, Todorovic R, Ristic M (1967) Infectious Diseases of Man and Animals. Vol 1. Academic Press, London.
- Figueroa JV, Chieves LP, Johnson GS, Buening GM (1992) Detection of *Babesia bigemina* infected carriers by polymerase chain reaction amplification. *J Clin Microbiol.* 30: 2576–2582.
- Ghai JK, Singh M, Singh A (2008) Population dynamics of ixodid ticks infesting cattle in Bathinda and Hoshiarpur districts in the Punjab State. *Annals Biol.* 24: 95–100.
- Hussein AH, Mohammed N Abd-EL-Salam, Mohammed HK (2007) Theileriosis and babesiosis in cattle: haemogram and some biochemical parameters. Proceedings of XIII International Congress of ISAH, June 17–21. Tartu, Estonia, pp. 143–150.
- Juyal PD, Kalra IS, Singla LD (1994) Prevalence of haemoprotozoans in domestic animals in Punjab. Proceedings of the 6th national congress of veterinary parasitology, 22–24 Oct 1994, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur.
- Kahn CM, Line S, Aiello SE (Eds) (2005) Reference Guides Table 7: Serum biochemical Reference Ranges. In: Merck Veterinary Manual 9<sup>th</sup> Edn, Merck and Co, Whitehouse station, USA, p. 89.
- Latimer KS, Mahaffey EA, Prasse KW (2005) Serum biochemical reference ranges. In: Kahn CM, Line S, Aiello SE (Eds.): Merck Veterinary Manual, 9<sup>th</sup> Ed, Merck and Co, White house station, USA, p. 2586.
- McLeod R, Kristjanson P (1999) Tick cost: economic impact of ticks and TBD to livestock in Africa, Asia and Australia. International Livestock Research Institute (ILRI), Nairobi, Kenya.
- Pandey N, Misra S (1987) Haematological and Biochemical response to haemolytic anaemia of clinical Babesiosis in cattle and therapy. *Ind Vet J.* 64: 882–886.
- Passos LM, Bell-Sakyi L, Brown CG (1998) Immunochemical characterization of in vitro culture-derived antigens of *Babesia bovis* and *Babesia bigemina*. *Vet Parasitol.* 76: 239–249.
- Ruprah MS (1985) A Text Book of Clinical Protozoology. Oxonian Press Pvt. Ltd, New Delhi.
- Sharma AK, Katoch RC, Nagal KB, Kishwaria RS, Sharma SK (2000) Bovine Babesiosis in Palam valley of Himachal Pradesh. *Ind Vet J.* 77: 731–732.
- Singh AP, Singla LD, Singh A (2000) A study on the effects of macroclimatic factors on the seasonal population dynamics of *Boophilus micropus* (Canes 1888) infesting the cross-bred cattle of Ludhiana district. *Int J Anim Sci.* 15 (1): 29–31.
- Singh J, Tuli A and Singla LD (2003) Mixed *Anaplasma* and *Babesia* infection outbreak in crossbred cattle. *Punjab Vet J.* 3(2): 73–74
- Thrusfield M (2005) Veterinary Epidemiology, second edition. Blackwell Science Ltd, UK.
- Vohara SP, Patel JV, Patel BB, Patel SB, Umale RH (2012) Seasonal incidence of haemoprotozoan diseases in crosses bred cattle and buffaloes in Kaira and



Anand districts of Gujarat, India. Vet World. 5: 223–224.  
Yeruham I, Avidar Y Aroch I, Hadani A (2003)

Intra-uterine infection with Babesia bovis in a 2-day- old calf. J Vet Med B Infect Dis Vet Public Health. 50: 60–62.