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Oxidative and haemato-biochemical alterations in theileriosis affected cattle from semi arid endemic areas of India

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Bovine tropical theileriosis (BTT) is the most common tick-borne disease of cattle principally caused by *Theileria annulata* and transmitted by tick *Hyalomma annatolicum annatolicum* in India. This intracellular protozoan parasite causes a severe, and often fatal, disease of pure and crossbred cattle in tropical and subtropical countries (Sudan *et al.* 2015) leading to reduced productivity and considerable mortality in cattle (Razavi *et al.* 2015). During the part of life cycle inside the erythrocytes, merozoites metabolize hemoglobin and produces free radicals which enhance oxidative stress in the infected animals (Hasanpour *et al.* 2013). Even though haemolytic anaemia is hallmark of the disease (Omer *et al*. 2002), its pathogenesis is not completely understood (Saleh *et al.* 2011). Some recent reports suggested that anaemia could be a consequence of the oxidative damage of RBCs by lipid peroxidation of the membrane of RBCs (Grewal *et al*. 2005), as well as considerable changes of erythrocytic antioxidant enzymes activities (Rezaei and Dalir-Naghadeh 2006, Razavi *et al.* 2015).

Therefore, the present study was intended to evaluate the oxidative and haemato-biochemical status as indicators of oxidative damage of the erythrocytes and their association with indices of anaemia in an outbreak of theileriosis in crossbred cattle.

Animals and samples for clinical investigation: A natural outbreak was reported in a herd of 200 cows reared in nearly identical nutritional and management conditions with history of fever, progressive weakness, anaemia and death of few animals. On clinical examination, the infected cows showed signs of theileriosis including dullness, anorexia, corneal opacity, enlargement of superficial lymph nodes, lacrimation, respiratory manifestations, nasal discharge, anaemia (paleness of mucous membranes) and fever (>40°C) along with various degrees of ticks infestation. Blood samples were collected in heparinised vials from 30 animals of the herd and aliquoted in two parts. The first part was used for hemato-biochemical evaluation while the second part was stored at —20°C for DNA extraction and PCR based diagnosis.

Diagnosis of theileriosis and haematological examination: Three thin blood films from every sample were prepared, fixed with absolute methanol (5 min), stained with 10% Giemsa solution (45 min) and examined under oil immersion (×1000), to observe abnormal red blood cells (RBCs) and intra-erythrocyte forms of *Theileria* sp. Besides this, DNA was extracted from each whole blood sample by traditional phenol chloroform method with minor modifications (Sudan *et al.* 2015) and confirmation of *T. annulata* infection was carried out by using Tams-1 gene based PCR primers as described by Sudan *et al.* (2016). The PCR products were separated by electrophoresis on 1.5% agarose gel.

The RBCs and WBCs counts were determined using a hemocytometer, whereas packed cell volume (PCV) and Hb concentration were determined by microhematocrit and cyanomethemoglobin methods, respectively (Jain 2000). Differential leukocyte counts were estimated manually, and the erythrocytes morphology was examined. The mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) were calculated using standard formula.

Estimation of oxidative and biochemical parameters: The plasma was harvested by centrifugation of blood samples at 2000 rpm for 10 min and buffy coat were removed. The obtained erythrocyte pellet was washed thrice with 0.15 M NaCl and further RBCs were suspended in PBS which was used for the estimation of lipid peroxidation

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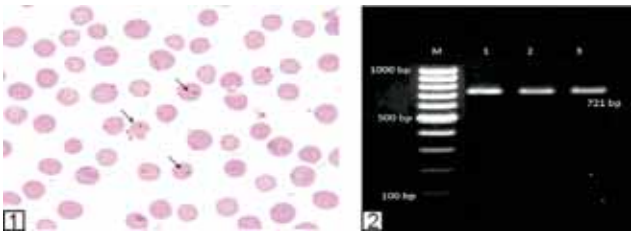
(LPO), superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione-s-transferase (GST) and reduced glutathione (GSH) using standard methodology (Pandey *et al.* 2015). All analyses of oxidative parameters were done as soon as possible after sample collection. The harvested plasma samples were analyzed for aspartate aminotransferase (AST), plasma total protein, albumin, globulin, albumin-globulin ration (AG ratio), total cholesterol, triglycerides and creatinine using commercially available kits (Cogent, Clinical Chemistry Division of SPAN Diagnostic Ltd, India).

Statistical analysis: The data obtained were analysed using Student's t-test with a P value of <0.05 considered as significant.

The adult animals aged between 2–5 years showing clinical symptoms of theileriosis, and confirmed on the basis of presence of intra-erythrocytic piroplasm stage of the protozoan parasite *T. annulata* (Fig. 1) in Giemsa stained thin blood film as well as presence of specific band at 721 bp (Fig. 2) using Tams-1 gene based PCR primers were considered as positive. While apparently and clinically healthy adult animals of same age group confirmed negative in all 3 blood smears as well as by PCR were considered as healthy control animals.



Figs 1–2.**1.** Microscopic examination of giemsa stained blood film showing piroplasms in erythrocytes. **2.** The electrophoretic pattern of PCR amplicon on blood samples using TAMS 1 gene primer of *Theileria annulata*, where lane M: DNA ladder, lane 1,2,3: positive samples for *T.annulata* at 721 bp.

Erythrocyte morphology and haematological findings: The infected cows revealed a wide range of morphologically abnormal RBCs including target red cells, bite cell, reticulocytosis, macrocytosis, and anisocytosis. In addition wrinkled (crenated) cells with many small sharp, blunt knob-like projections of uniform length were also found evenly distributed around the cell periphery (echinocytosis). The RBCs of control healthy cows appeared normal without deformability. The values of hematological indices in infected and control healthy animals are presented in Table 1. The study revealed significant fall in TEC, Hb concentration, PCV, MCH and MCHC in the infected (P<0.01) compared to healthy control cows. The cows infected with theileriosis showed a significant rise in TLC and neutophils, while decline in lymphocytes and monocytes compared to healthy animals.

Oxidative and biochemical status: The results of the oxidative and biochemical status of *T. annulata* affected and healthy cows are depicted in Table 1. The findings

Table 1. Levels of oxidative and haemato-biochemical indices in healthy and *Theileria* infected cattle

Parameter	Infected cows (n=10)	Healthy cows (n=5)
Lipid peroxidation (nM MDA/ml packed RBCs)	5.65±0.20*	2.25±0.07
Superoxide dismutase (U/mg protein)	9.09±0.36*	4.85±0.16
Catalase (mM H_2O_2 utilized/ min/mg protein)	5.00±0.29*	2.44±0.16
GST (µM/min/mg Hb)	1.16±0.045*	1.57±0.03
GSH (mM /ml packed RBCs)	3.16±0.22*	5.20±0.67
Aspartate transaminase (IU/L)	59.58±8.88*	14.66±1.03
Total protein (g/100 ml)	6.57±0.28	7.76±0.19
Albumin (g/100 ml)	2.40±0.15	3.24±0.14
Globulin (g/100 ml)	4.17±0.26	4.51±0.30
AG ratio	0.60±0.06	0.73±0.07
Total cholesterol (mg/100 ml)	84.04±5.93	55.84±4.09
Triglycerides (mg/100 ml)	25.65±2.14	20.97±1.93
Creatinine (mg/100 ml)	1.05±0.12	1.25±0.14
Haemoglobin (g/100ml)	8.72±0.26*	13.20±0.22
TEC (million/µL)	5.09±0.07*	6.46±0.12
PCV (%)	25.28±0.76*	42.24±0.72
MCV (fL)	44.76±0.43*	41.79±0.58
MCH (pg/L)	12.17±0.07*	16.92±0.86
MCHC (g/dl)	27.20±0.23*	40.47±1.91
TLC (10^3/µL)	9.53±0.09*	7.43±0.09
Lymphocytes (%)	43.16±0.48*	59.62±0.67
Neutrophil (%)	53.00±0.92*	34.12±0.74
Eosinophils (%)	3.58±0.60	4.12±0.22
Monocytes (%)	1.00±0.25	2.37±0.18

*indicate significant difference between groups.

demonstrated elevated levels of LPO and AST, and significant reduction in activity of SOD, CAT, GST and levels of GSH in cows infected with *T. annulata* as compared to healthy control animals. The haematological indices indicating anaemia (Hb, TEC, PCV, MCH and MCHC) showed negative correlation with LPO and positive correlation with oxidative indices (SOD, CAT, GST and GSH) (Table 2). In biochemical indices, AST activity

Table 2. Correlation between markers of oxidative stress and anaemia in cattle affected with theileriosis

	LPO	SOD	CAT	GST	GSH	Hb	TEC	PCV	MCV	MCH	MCHC
LPO	1.00										
SOD	-.844**	1.00									
CAT	-.508*	.601**	1.00								
GST	-.566**	.765**	.753**	1.00							
GSH	-.592**	.528*	.646**	.608**	1.00						
Hb	-.819**	.929**	.698**	.816**	.605**	1.00					
TEC	-.757**	.863**	.751**	.784**	.629**	.929**	1.00				
PCV	-.742**	.869**	.617**	.777**	.610**	.832**	.787**	1.00			
MCV	-0.33	0.41	0.14	0.35	0.27	0.28	0.12	.705**	1.00		
MCH	-.813**	.926**	.646**	.805**	.570**	.989**	.867**	.830**	0.35	1.00	
MCHC	-.727**	.811**	.627**	.703**	.495*	.936**	.869**	.584**	-0.04	.920**	1.00

*indicate significant difference between groups.

showed higher levels in infected animals compared to healthy animals. However slight rise in total plasma protein, albumin, globulin, cholesterol and triglycerides and slight decline in creatinine were observed in infected animals than healthy animals, but the variations were non-significant.

In present study the blood smear examination confirmed the presence of piroplasm in the erythrocytes of infected cattle. In addition the erythrogram of infected cows showed significant decrease in Hb, PCV, MCH and MCHC and increase in MCV indicating macrocytic and hypochromic anaemia in affected cattle. Based on reference values for the PCV, most of the infected cattle were considered as mild to moderately anaemic (PCV 22–24) (Carlson 1990). A variety of morphologically abnormal RBCs were also observed in the present study including anisocytosis, reticulocytosis, target red cells, bite cells, basophilic stippling, echinocytosis and polychromasia in anaemic animals. This phenomenon indicated the regenerative or compensatory responses of bone marrow to anaemia in infected animals (Razavi *et al*. 2015). Significant leukocytosis with lymphopenia and neutrophilia were observed in infected cattle compared to control healthy animals in present study. The stimulation of lymphoid tissues and stem cells in the bone marrow by the parasite and their toxins may have resulted into leukocytosis (Youssef *et al*. 2015). The release of endogenous corticosteroid due to acute disease or stress and or inflammation accounted for significant neutrophilia observed in infected animals (Feldman *et al.* 2000). While lymphopenia recorded in infected cattle may be due to lysis of lymphocytes caused by release of protozoal merozoites into blood stream (Feldman *et al.* 2000) and infiltration into various organs (Omer *et al.* 2002).

In addition, theileriosis affected cattle revealed significant rise in LPO (MDA) and significant fall in SOD, CAT, GSSH and GST. Besides, markers of anemia (Hb, TEC, PCV, MCV, MCH and MCHC) revealed strong inverse relationship with lipid peroxidation and positive correlation with markers of antioxidants signifying that the invasion of RBCs by the parasites could affect key antioxidant defense barriers and causing lipid peroxidation resulting in significant RBC damage and finally leading to extravascular hemolysis and haemolytic anaemia. Erythrocytes of infected animals are continuously exposed to free radicals (FR) such as O_2^-, NO, OH and H_2O_2 of intra-erythrocytic origin (toxic action of parasites in the erythrocyte cell) (Al-Emarah *et al*. 2012) and extra-erythrocytic origin from the proinflammatory cytokines activated neutrophils and/or macrophages (Ahmed *et al.* 2008). The overproduction of free radicals (FR)/reactive oxygen species (ROS) bring about peroxidation of membrane lipid and gave MDA as the finished product, which is the major reactive aldehyde resulting from the peroxidation of biological membranes (El-Far *et al.* 2014). Enhanced MDA concentration along with decreased levels of SOD, CAT, GSH, and GST in theileriosis infected cattle indicate that antioxidant defence mechanisms were not sufficient to neutralize the oxidative stress (Grewal *et al.* 2005), and consequent lipid peroxidation through the production of ROS and FR damages vital components of the cell, including proteins and lipids (Murray *et al.* 2003) lead to oxidative deleterious damage in cells (Friedman 1979). Further, oxidative injury to erythrocyte causes loss of fluidity, increased permeability to ions, which lead to a decrease in membrane potential, stability and function (Kanias and Acker 2010). Additionally highly reactive MDA molecule shown to cross-link RBC phospholipids and proteins, affecting membrane fluidity and impairing various membrane functions, which causes a decrease in RBC survival (Sugihara et al.1991). Consequently, this process can reduce membrane symmetry and increased membrane permeability; leading to morphological changes in the RBC cell surface (Saluja *et al.* 1999). In addition, Rezaei and Dalir-Naghadeh (2006) reported that membrane proteins may be more susceptible to oxidative damage and more linked to anemia than membrane lipids and Hb of *T. annulata* infected calves. Bracci *et al.* (2002) reported that denaturation of membrane proteins generally precedes LPO in the erythrocyte. Further, the oxidative attack on cytoskeletal proteins, rather than on membrane lipids, is the most contributing factor for morphologic alteration of the erythrocyte (echinocytosis). Protein modification in the

erythrocytic membrane affects the elastic properties of cytoskeleton and the maintenance of the surface area (Pantaleo *et al*. 2010).Thus structural alteration in erythrocytes is likely to increase their propensity towards haemolysis by the process of splenic uptake of erythrocytes damaged during the course of oxidant-induced haemolytic anemia (McMillan *et al.* 2005).

Significant rise in AST activity and no change in other biochemical indices were observed in present experiment. The significant rise in AST activity may be attributed to soft tissue damage such as muscles and liver (Youssef *et al.* 2015). No significant differences in total cholesterol and triglycerides were noticed between theileriosis affected and healthy animals. These findings of lipid profile were corroborated with the reports of Razavi *et al.* (2015). Besides, no significant changes were found in the plasma total protein, albumin, globulin and creatinine concentrations between infected and healthy cattle.

Taking together, the notable decline in the levels of antioxidant and the considerable rise in the lipid peroxidation and consequent haemolytic anaemia imply that *T. annulata* infection may impede the defending antioxidant mechanisms of RBCs against oxidative injuries and the antioxidant enzymes were not able to avert oxidative damage to the RBCs membrane. This oxidative damage is also linked with the morphological changes of the erythrocyte that significantly contribute in oxidant-induced haemolytic anemia.

SUMMARY

Blood samples were collected from 30 confirmed theileriosis affected adult cows belonging to a single herd. The infection of theileriosis in suspected and healthy cows was confirmed based on clinical signs, blood smear examination and PCR based assay. The collected blood samples were evaluated for levels of Hb, PCV, MCV, MCH, MCHC, DLC, LPO, GSSG, SOD, CAT, GST and AST. The results revealed substantial decrease in levels of Hb, PCV, MCH, MCHC, lymphocytes, SOD, CAT, GST and GSH and increase in LPO, AST, and neutrophils in infected animals compared to healthy animals. The association observed between enhanced erythrocytic oxidation and reduction of hematological indices suggests that antioxidant mechanisms of erythrocytes that protect them against oxidative damage may be disturbed during theileriosis which lead to erythrocytic destruction and progression of the anaemia in *T. annulata* infection in cows.

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