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RESEARCH ARTICLE

Effect of Equine Piroplasmosis on Hematological and Oxidative Stress Biomarkers in Relation to Different Seasons in District Sargodha, Pakistan

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A B S T R A C T

Equine piroplasmosis is an endemic tick-borne disease of horses and other equids that is caused by *Theileria equi* and *Babesia caballi*. The study was aimed to investigate the seroprevalence of equine piroplasmosis during different seasons at district Sargodha located in central Punjab, Pakistan. For the detection of seropositive animals among sampled horses, the competitive enzyme-linked immunosorbent assay (cELISA) was performed against samples collected periodically during the four seasons of the year. On average basis, overall 36% of sampled horses were found seropositive with *T. equi* infection. Following the evaluation of seropositivity, subsequent estimation of oxidative stress biomarkers indicated that during winter season, total oxidant status (TOS) was found significantly ($P \leq 0.05$) increased while total antioxidant capacity (TAC), arylesterase and paraoxonase activity were found significantly decreased ($P \leq 0.05$) in seropositive horses as compared to healthy horses. Moreover, hematological parameters including red blood cells count, white blood cells count, MID and MID% were found significantly ($P \leq 0.05$) decreased while platelets, number of lymphocytes, number of granulocytes, and the percentage of lymphocytes were found significantly ($P \leq 0.05$) increased in seropositive horses as compared to seronegative horses in different seasons. In conclusion, we found that equine piroplasmosis markedly affect the oxidative stress biomarkers in seropositive horses primarily during the winter season as compared to all other seasons highlighting the seasonal impact of the disease.

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INTRODUCTION

In Pakistan, the livestock sector engages about 30-35 million people to raise their livelihoods and undoubtedly playing a significant role in supporting the rural economy by contributing 35-40% to their earnings (Shahnawaz *et al*., 2011). Livestock Census Punjab, Pakistan has reported a population of 4,190 horses out of 74,204 total heads of equine population (including horses, donkeys, and mules) in the city of Sargodha located in the central part of Punjab, Pakistan (Anonymous, 2018). The district Sargodha has wide ranged climate extremes, with a temperature range of maximum 50°C (122°F) in the summer season to a minimum of -1°C (30.2°F) in winter season. Moreover, a heavy rainfall is accompanied to the monsoon season starting from mid-June to mid-August. Under these harsh and extreme conditions of Sargodha, the use of equines as a cheap carriage for industrial and agricultural supplies and products is quite common. In literature archive, very less information is available regarding the prevalence of equine piroplasmosis in Pakistan. In particular, no study has ever been conducted on the prevalence of piroplasmosis with respect to different seasons of the year and oxidative stress index in seropositive equids.

Equine piroplasmosis (EP) is a disease of equids including horses, donkeys, mules and zebras that primarily caused by *Babesia caballi* and/or *Theileria equi*.

Both the protozoans are haemoparasites and mainly transmit by ixodid ticks. In equines, the tick infestation has been reported as a serious threat to the economic growth of livestock industry (Ghosh *et al.*, 2007, Qablan *et al.*, 2013). Among both causative agents, the *Theileria equi* is considered primarily more pathogenic as it exhibits a more severe and acute form of equine piroplasmosis.

Several epidemiological studies have demonstrated an association of equine piroplasmosis with tick infestation (Kerber *et al.,* 2009), age range (Qablan *et al.,* 2013) extensive farming (Ribeiro *et al.,* 2013), geographic area and gender (Moretti *et al.,* 2010). All of these important factors need to be taken in account while addressing the prevention and control of the vector borne diseases (Kerber *et al.,* 2009; Abbas *et al*., 2014). However, the equine piroplasmosis was never studied in context to the seasonal variation as it could influence the tick prevalence as well in the premises and impact of seropositivity on the oxidative stress in the animals as a carrier state.

The infected animals remain carrier and serve as potential source of infection to the ticks (vector), which infest readily parasites to the equid hosts (Sumbria *et al.,* 2014). However, recovered animals from acute infections become asymptomatic but sustain sub clinical infections (Perez-Llaneza *et al.,* 2010; Schneider *et al.,* 2011). In such scenario, infested animals apparently looking normal still remain a potential source for vector to transmit the disease naturally. Oxidative stress plays pivotal role in the pathogenesis of parasitic infections (Crnogaj *et al.,* 2010). Oxygen free redicals mainly target fatty acids in membrane resulting modifications in protein, nucleic acid and cell integrity (Muller *et al*., 2003).

The objective of the current study was to study the oxidative stress biomarkers in sub-clinical equine piroplasmosis during different seasons of the Sargodha region of Pakistan. A sensitive molecular tool, cELISA was used to detect *T. equi* and *B. caballi* in horses. Furthermore, the present study provided a baseline data regarding the presence of *T. equi*, *B. caballi* and different risk factors in different seasons involved in the spread of babesiosis in equine. We also compared the effect of different seasons on various hematological and serum biochemical parameters between seropositive and seronegative horses to demonstrate the effect of babesiosis on the blood profile of the host.

MATERIALS AND METHODS

Experimental design: A total of 50 horses with different body weight (200-450kg bodyweight) and ages (04-21years) kept at a private farm were selected for the current study. Apparently healthy-looking horses were randomly selected for the current study. These animals were properly fed on grazing, alfalfa fodder and supplementation of chickpea (3-5kg/day). All horses on the farm were raised under humidity and temperature-controlled environment that was well monitored during the course of study. The sampling was performed for a period of one year starting from autumn-2017 to summer-2018. Blood samples were drawn periodically from all fifty horses once during the peak phase of each season (autumn, winter, spring, and summer). All blood samples were collected in anticoagulant lacking sterilized, chilled vacutainers. The serum samples were then saved in collection tubes and transported to research laboratory under controlled temperature conditions and subsequently saved at -20°C until further analysis.

Laboratory investigations: After properly thawing the serum samples, cELISA was performed for the detection of seropositivity against *Theileria equi* and *Babesia caballi* using commercially available antibody kits by Veterinary Medical Research and Development, Pullman, WA 99163 USA (catalog number: 274-2 and 273-20). These assays target serum antibodies against antigen EMA-1 in *T. equi* merozites and RAP-1 antigen in *B. caballi* merozites respectively. Optical density (OD) was measured by ELISA plate reader (Biorad®). Final results were obtained using formula:

Inhibition (%) =100 [1-(Sample OD ÷ NC OD)]

Samples showing inhibition (%) ≥40 were considered seropositive while sample with ≤40% were regarded as seronegative.

Following the evaluation of seropositivity, the serum samples were used to estimate the oxidative stress biomarkersincluding total oxidant status (TOS), total antioxidant capacity (TAC), paraoxonase and arylesterase enzyme activity to estimate the impact of piroplasmosis on oxidative stress index. Both total oxidant status and total antioxidant capacity were measured by calorimetric method explained by Anwar *et al.* (2012). The TOS of samples was determined in appropriate equivalence of H_2O_2 standards from the standard curve. Likewise, to evaluate the total antioxidant capacity (TAC; mmol of Trolox equivalent L^{-1}), the appropriate standards were made from Trolox (vitamin E analog; 30 mM stock) to construct the standard curve. The minimum detectable range of this assay was 0.18 mmol L^{-1} and was linear up to 6 mmol of Trolox $_{equiv.}$ L^{-1} with intra-assay CV below 3%. The arylesterase enzyme activity was estimated by the method explained by Juretić *et al.* (2006). For the measurement of paraoxonase activity the method explained by Juretić *et al.* (2006) was implemented.

In addition to the evaluation of oxidative stress biomarkers from serum samples, blood samples were also evaluated for hematological profile to understand the general health status of seropositive and seronegative animals. The parameters include RBC (Red Blood Cells) count, MCV (Mean Corpuscular Volume), RWD (Red Blood Cell Distribution Width), HCT(Hematocrit), PLT (Platelet Count), MPV (Mean Platelet Volume), WBC (White Blood Cells) count, HGB (Haemoglobin), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), LYM (Lymphocyte Count), GRAN (Granulocytes Count), MID (cells include less frequently found), LYM% (Lymphocyte percentage), GRAN% (Granulocyte percentage) and MID% (percentage of cells include less frequently found). All hematological parameters were estimated using Hematology Analyzer (Model. No. D×H 900) in the Health Biology and Metabolic Disorder Laboratory, Department of Physiology, Government College University, Faisalabad.

Statistical analysis: Logistic regression was performed to find Odd Ratio, P value and 95% confidence intervals among sex and age in prevalence data using SPSS.17. Homser-Lemeshow test, Negelkerke R square test were used to assess the fitness of model. All datasets were expressed as mean ± SEM and statistical significance among groups was determined by using student T test and one-way ANOVA by Graph Pad Prism 6.0. followed by post hoc test through software CoStat version 6.45. The results were considered statistically significant at P≤0.05.

RESULTS

Prevalence of *Theileria equi:* According to cELISA analysis performed for the detection of seropositivity against *Theileria equi* and *Babesia caballi*, on average, 36% of sampled horses were found seropositive with *T. equi* infection (Table 1). However, none of the fifty sampled horses were found seropositive with *B. caballi* vector throughout the study period (autumn, 2017 to summer, 2018). Subsequent analysis of serum samples for the oxidative stress biomarkers indicated that mean serum TOS level (µmol of $H_2O_{2\ equiv.}\ L^{-1}$±SEM) was significantly (P≤0.05) increased in seropositive samples particularly in winter season followed by the autumn season as presented in Fig. 1. Overall mean serum TOS level was significantly (P≤0.05) increased in winter as compared to all other seasons in all horses irrespective of seropositivity (Fig. 2). Mean TAC level (mmol of Trolox $_{equiv.}\ L^{-1}$±SEM) was significantly (P≤0.05) decreased in seropositive horses in comparison to seronegative horses in all seasons. However, that decrease was more evident in the winter season as compared to other seasons as given in Fig. 3. However mean TAC level was significantly (P≤0.05) decreased in summer as compared to other seasons irrespective of seropositivity as shown in Fig. 4.

Similarly, mean arylesterase activity (kU $min^{-1}ml^{-1}$) was found significantly (P≤0.05) decreased in seropositive horses in comparison to seronegative horses during winter season followed by summer, autumn and spring seasons as depicted in Fig. 5. Moreover, paraoxonase activity (kU $min^{-1}ml^{-1}$) was found significantly (P≤0.05) decreased in seropositive horses in winter in comparison to seronegative horses followed by summer, autumn and spring seasons as indicated in Fig. 6. Overall, paraoxonase activity was found significantly (P≤0.05) decreased during winter season in comparison as compared to all other seasons while arylesterase activity was significantly (P≤0.05) decreased in winter season as compared to other seasons irrespective of seropositivity presented in Fig. 7.

Statistical analysis of hematological parameters (Table 2) revealed that RBC count was decreased significantly (P≤0.05) in autumn while WBC count was decreased significantly (P≤0.05) in spring and MID% was found decreased significantly (P≤0.05) in all seasons in seropositive horses as compared to healthy horses. GRAN was found decreased significantly (P≤0.05) in all seasons except autumn in seropositive horses as compared to healthy horses. The MCV, RWD, MID, MPV, HCT, HGB, MCH and MCHC values did not differ significantly (P≥0.05) in seropositive and healthy horses throughout the year i.e., during autumn, winter, spring and summer. Platelet count got increased significantly (P≤0.05) in winter, LYM was significantly (P≤0.05) increased in summer, LYM% was increased significantly (P≤0.05) in winter and summer and GRAN% was increased significantly (P≤0.05) in all seasons except winter in seropositive horses as compared to healthy horses.

DISCUSSION

In current study, overall about 36% of horses were found seropositive with protozoan *T. equi* which in accordance to the results of Nagore *et al.* (2004) who pointed out 37.5% prevalence of theileriosis (*T. equi*) and babesiosis (*B. caballi*) of 20.81% in horses during August and November 2002 in Northern region of Spain. Alanazi *et al.* (2012) reported seroprevalence of *T. equi* and *B. caballi* as 16.5 and 8.8% respectively in horses in central part of Saudi Arabia. Al-saad *et al.* (2010) also detected Babesia species through cELISA thus confirming 78.3% prevalence of babesiosis in horses. Ros-Garcia *et al.* (2013) observed piroplasm 12.5% prevalent in horses. Ribeiro *et al.* (2013) also confirmed the prevalence of *T. equi* in 17.9% of horses. Shehzad *et al.* (2003) reported 5.06% positive samples for Babesia species out of which 27.27% samples were positive with mixed infection of *T. equi*. Garba *et al.* (2011) reported prevalence of *T. equi*

Table 1: Percentage of *Theileria equi* positive samples of equine serum (n=50) evaluated in different seasons

		Total tested	Seropositive				Overall average seroprevalence (%)	Odd ratios (CI 95%)	P value
			Autumn	Winter	Spring	Summer			
	Total	50	10	24	25	22	18(36)		
Sex	Male	19	0	21	5	5	4(21.05)	0.346(0.092-1.303)	0.117
	Female	31	16	25	35	35	13(41.9)	1	
Age	≤10 years	32	15	25	20	26	13(40.62)	1.507(.413-5.503)	0.534
	≥10years	18	5	22	50	22	5(27.77)	1	

Table 2: Hematological parameters (Mean ± SEM) in serum samples of healthy and seropositive horses in different seasons

Seasons/	Autumn		Winter		Spring		Summer	
Parameter	H	P	H	P	H	P	H	P
RBC (10^{12}/L)	5.24±0.21	4.93±0.11*	6.53±0.31	6.20±0.41	6.40±0.11	6.12±0.13	6.67±0.22	6.99±0.14
WBC (10^9/L)	8.49±0.11	7.82±0.3	8.6±0.11	8.8±0.55	9.58±0.44	7.41±0.41*	9.36±0.54	10.22±0.33
PLT (10^9/L)	67.04±3.0	69.2±0.4*	121.8±0.46	169.1±1.22**	97.30±1.51	71.5±1.48	116.68±1.49	128.2±2.06
LYM (10^9/L)	5.04±0.3	4.46±0.4	2.90±0.5	3.19±0.54	5.39±0.14	4.71±0.33	6.94±0.52	8.89±0.59*
GRAN (10^9/L)	1.35±0.31	1.42±0.1	1.73±0.08	1.98±0.23	0.83±0.11	0.52±0.04**	0.49±0.21	0.39±0.012*
MID (10^9/L)	2.92±0.4	2.14±0.14*	4.05±0.22	3.06±0.21*	3.45±0.29	2.22±0.22*	1.98±0.1	1.51±0.23*
RWD (%)	17.95±.022	18.3±0.9*	21.90±0.41	21.64±0.22	21.2±1.01	21.18±1.1	21.36±1.33	21.10±1.21
LYM%	53.44±2.3	52.7±3.1	33.51±0.24	38.85±2.04*	58.17±1.04	59.79±2.05	71.49±2.14	78.8±2.34*
GRAN%	9.15±0.12	18.9±1.8**	21.12±0.65	22.46±1.05	5.8±1.45	6.83±0.31*	5.4±0.11	6.61±0.13*
MID%	31.15±0.4	27.3±0.52*	47.48±0.66	36.13±1.05*	36.37±1.45	26.7±1.05*	22.38±0.99	14.3±0.73**

H= Healthy (Seronegative), P=Seropositive with *T. equi*, *=P<0.05, **=P<0.01.

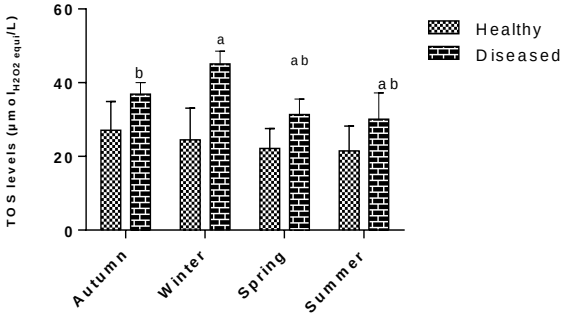

Fig. 1: Mean TOS concentration (µmol H_2O_2 equivalent L^{-1}±SEM) measured in different groups; healthy (Seronegative) and (Diseased) seropositive in all seasons.

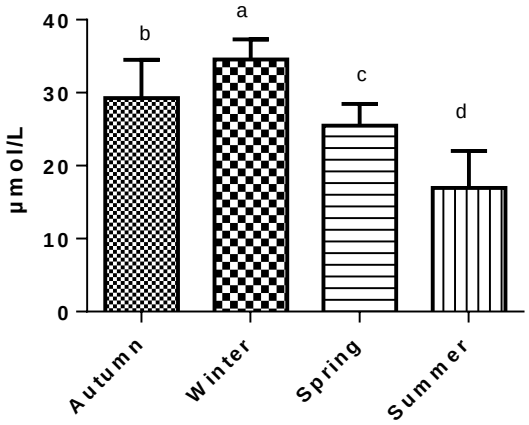

Fig. 2: Overall mean TOS concentration (µmol H_2O_2 equivalent L^{-1}±SEM) measured in different seasons irrespective of health status.

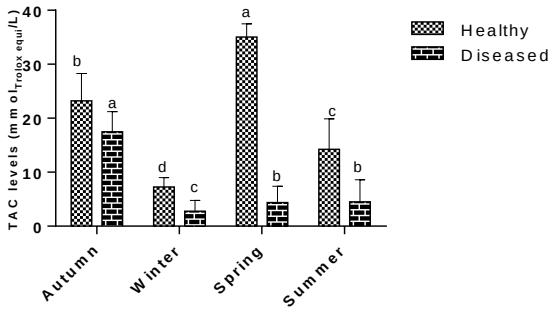

Fig. 3: Mean serum TAC (mmol of Trolox equivalent L^{-1}±SEM) measured in different groups; healthy (Seronegative) and (Diseased) seropositive in all seasons.

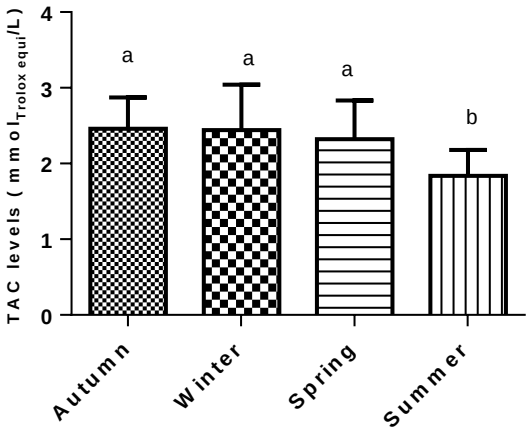

Fig. 4: Overall mean serum TAC (mmol of Trolox equivalent L^{-1}± SEM) measured in different seasons irrespective of health status.

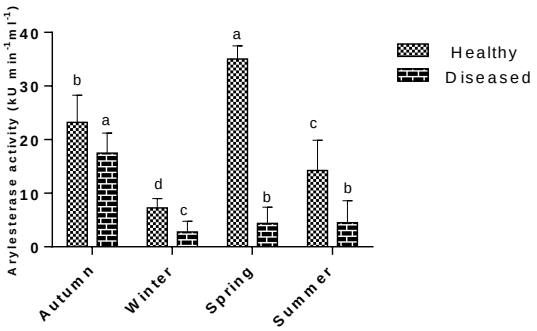

Fig. 5: Mean serum arylesterase (kUmin^{-1}ml^{-1}±SEM) measured in different groups; healthy (Seronegative)and (Diseased) seropositive in all seasons.

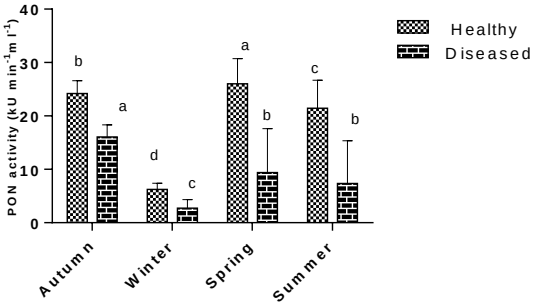

Fig. 6: Mean serum paraoxonase (kUmin^{-1}ml^{-1}±SEM) measured in different groups; healthy (Seronegative)and (Diseased) seropositive in all seasons.

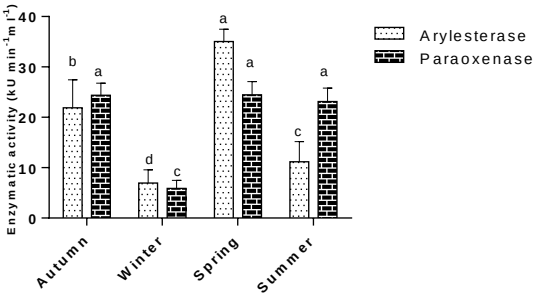

Fig. 7: Overall mean serum arylesterase and paraoxonase (kUmin^{-1}ml^{-1}±SEM) measured in different seasons irrespective of health status.

(80.4%) in overall equine samples in Niger. Mekibib *et al.* (2010) found *T. equi* (71.43%) in donkeys between months of November 2008 and March 2009 in Ethiopia. Sgorbini *et al.* (2015) pointed out prevalence of 56.52% *T. equi* in equines of central region of Italy during the spring and early summer. We found a seroprevalence of theileriosis as 36% that in accordance to the results of Sigg *et al.* (2010) who reported prevalence of *T. equi* 27.3% in Spain and 25% in Portugal. None of the sample in current was found positive with *B. caballi*. High prevalence of *T. equi* as it is more pathogenic and frequently detected than *B. caballi* (Ribeiro *et al.*, 2013).

In current study, prevalence of *T. equi* was higher in females (41.9%) than male (21.05%) horses. Results of Sigg *et al.* (2010) less associate with present study which shows that prevalence of *T.equi* was higher in male (6.2%) horses than females (4.4%). However, prevalence of theileriosis in female horses (41.9%) in current study are in close agreement with Heuchert *et al.* (1999) reporting prevalence of babesiosis was 38.1% in female horses and Machado *et al.* (2012) depicting theileriosis

(31.81%) and babesiosis (20.45%) in donkeys. In present study highest prevalence of *T. equi* was reported in spring (25%) followed by winter (24%), summer (22%) and autumn (10%) Salib *et al.* (2013) reported highest prevalence of theileriosis in summer (25.81%) which correlate current study. In current study, prevalence of *T. equi* vary differently in different seasons, however highest prevalence of vector was reported in spring season (25%). While Female (35%), horses with age group ≥10 years (50%) and pregnant females were found seropositive (38%) in spring season. Tick infestation is the main source of transmission of Babesia species in equine (De waal, 1992). Highest tick prevalence in spring and summer correlate the highest prevalence of theileriosis in current study. Less prevalence of disease during autumn might be due to better management by the owner in the areas.

In current study, TOS level was significantly high in seropositive horses as compared to healthy horses in winter and autumn while nonsignificant in normal healthy horses in all seasons. Oxidative stress plays a key role in the progression of infections (Mandas *et al.,* 2009). High level of free radicals in seropositive horses is due to parasitic burden. In aerobic environment, the synthesis of free radicals (ROS) is an inevitable outcome. Through the process of lipid peroxidation encompassing the oxidation of biological membranes and lipoproteins, the production of free radical hydroxyl ions including superoxide anions and hydrogen peroxide occur. Results of current study correlate with Otsuka *et al.* (2002) showing rise in free radical synthesis in *B. gibsoni* infected dogs. TAC level was significantly decreased in overall samples in summer and was significantly decreased in winter in seropositive horses as compared to healthy horses. Arylesterase and paraoxonase (PON) activity was significantly decreased in overall samples and seropositive horses in winter season in seropositive horses as compared to normal healthy horses. Poor management of diet by the owner, frequent humid environment, harsh cold weather and shortage of natural fodder lead low TAC level in winter and fragile immunity in seropositive horses.

Platelet count, lymphocytes (LYM), LYM% and GRAN% were increased in different seasons in seropositive horses as compared to normal healthy horses. The RBC, WBC, GRAN, MID and MID% was decreased significantly in different seasons in seropositive horses as compared to normal healthy horses. Hemolytic anemia is resulted from infectious parasites *B. caballi* and *T. equi* in RBC's. Conformational changes in membrane protein and lipid content of the erythrocyte lead to reduced blood flow in microvasculature (Wise *et al*., 2013). Shehzad *et al.* (2003) reported in that in winter hematological parameters; RBC, WBC, HGB decreased while lymphocytes were decreased significantly in seropositive horses as compared to normal healthy horses. In current study HGB was nonsignificant in seropositive and healthy horses in all seasons showing a pre-erythrocyte stage (Mehlhorn *et al.,* 1993) that is in contrast to the results of Zobba *et al.* (2008) where HGB level was decreased in seropositive equine.

In previous study (Mahmoud *et al.,* 2016) the most frequent piroplasmosis induced hematological alteration resulted in decrease in erythrocyte count, Platelets, HCT, HGB, MPV and PDW with increased values of MCV. Parasitemia increases malondialdehyde in the plasma resulting in the frequent oxidative ions which is a product of lipid peroxidation that alters the RBC biochemical properties leading to hemolysis of RBC's (Ambawat *et al.,* 1999). Such hemolytic progression leads to reduction in the packed cell volume (PCV), hemoglobinuria and icterus in *T. equi* infection (Rothschild, 2013).

Conclusions: Our study highlights the prevalence of subclinical piroplasmosis in horses during the different seasons of the year in Pakistan. As anticipated, *Theileria equi* seropositive horses markedly affect the oxidative stress biomarkers primarily during the winter season highlighting the seasonal impact of the disease in context to oxidative stress.

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Authors contribution: This manuscript is based on PhD thesis of first author. HA and MA being the major supervisors and GH, SI and MS are the members of supervisory committee have conceived and designed the study. All the other authors have contributed in conducting analysis and writing the manuscript. All authors are involved in discussing the contents of manuscript and declare no conflict of interest.

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