



A STUDY ON MOLECULAR SURVEILLANCE OF *Theileria* spp. INFECTION AND ITS IMPACT ON HEMATOLOGICAL AND BIOCHEMICAL CHANGES IN NATURALLY INFECTED SMALL RUMINANTS AT MULTAN, PAKISTAN

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ABSTRACT

The current investigation was conducted to evaluate hematological and biochemical changes in naturally infected sheep and goats with *Theileria* spp. infection and to compare the conventional microscopic method of diagnosis with PCR amplification. A total of 200 blood samples (144 sheep and 56 goats) were collected and examined for the presence of *Theileria* infection. 12.5% and 39.5% blood samples were found positive with *Theileria* infection by microscopic examination and PCR amplification respectively. Hematological parameters were analyzed by automatic hematology analyzer (Diatron Abacus) while serum biochemistry was studied by Semi-automatic Chemistry Analyzer (Micro Lab 300). Hematological parameters revealed significant ($p < 0.05$) decrease in RBCs counts, Hb contents, PCV, MCH and MCHC values while non-significant ($p > 0.05$) correlation in MCV values of *Theileria* infected small ruminants. Biochemical analysis revealed significant ($p < 0.05$) reduction in total serum protein and albumin concentration while non-significant ($p > 0.05$) rise in urea and cholesterol level in infected animals compared to normal. *Theileria* piroplasms prevalence during early stage of disease without any clinical symptoms can only be identified through PCR amplification compared to microscopic examination.

Keywords: hemato-biochemical values; Pakistan; PCR; Small ruminants; *Theileria* infection.

INTRODUCTION

Theileriosis caused due to *Theileria* spp. (Piroplasmida, Theileridae) is a tick-borne protozoan disease found both in wild and domestic ruminants. *Theileria* genus belongs to Apicomplexa phylum including Babesia, Toxoplasma, Neospora, Plasmodium and others Radostits *et al.* (2007). Theileriosis transmitted by ticks, caused high morbidity and mortality in infected animals resulted in higher economic losses in tropical and subtropical regions of the world Aktas *et al.* (2005) and Rymaszewska *et al.* (2008). A total of six *Theileria* species caused ovine and caprine theileriosis from which *Theileria lestoquardi* considered highly pathogenic while *T. ovis* caused subclinical infection in sheep and goats Telmadarraiy *et al.* (2012). *Theileria* infection can be acute, subacute or chronic. Malignant ovine theileriosis (MOT) caused due to *T. lestoquardi* infection resulted in higher mortality in sheep and goats Rymaszewska *et al.* (2008), 100% mortalities had been reported in case of malignant ovine theileriosis Tageldin *et al.* (1992). *Theileria lestoquardi* causes fever, emaciation, lymphadenopathy, wasting, malaise anorexia, rapid heartbeat, dyspnea, listlessness, anemia, icterus, jaundice, pyrexia, intermittent diarrhea or constipation, weakness and termination of rumination Sayin *et al.* (2009). During theileriosis, *Theileria* schizonts are frequently observed in liver, spleen, lungs, kidneys and lymph nodes Hong *et al.* (2002). Clinical symptoms of theileriosis could be electrolyte imbalance, overdosing with calcium, digoxin, and cardiomyopathy Tehrani *et al.* (2012).

The diagnosis of *Theileria* spp. in general is based on microscopy of stained smears and presence of clinical based symptoms in animals infected with ovine theileriosis. But the above cited methods are beneficial only in acute cases of theileriosis and insufficient for detection of piroplasms in carrier animals Almeria *et al.* (2001) and Inci *et al.* (2010). In recent years, the molecular technique Polymerase chain reaction (PCR) is frequently used for detection of ovine and caprine piroplasms. Hematobiochemical indices had been employed in attempts to get information regarding health status, performance and fitness of animals. An aberration from normal values might serve a guide for the differential diagnosis of a disease status Mal *et al.* (2001). Blood glucose and albumin level reduced significantly while serum protein and creatinine level decreased non-significantly during theileriosis Baghshani *et al.* (2011). Biochemical profile of *Theileria* infected animals showed higher level of urea, aspartate amino transferase, bilirubin (end product of hemoglobin) level and lactate dehydrogenase compared to healthy animals Singhet *et al.* (2001). The significant decrease in RBCs counts, Hb contents and PCV values were identified while no changes found in MCH and MCHC values in infected goats with theileriosis Zangana *et al.* (2011). Hematobiochemical changes reported in earlier studies in infected small ruminants was based on experimental animals, the current investigations was designed to delineate hematobiochemical changes in naturally infected small ruminants with theileriosis which can be utilized for



diagnosis, prognosis and for better management of metabolic health status of infected animals.

MATERIALS AND METHODS

Study area

Multan is situated in Punjab province (southern region), Pakistan between 29°-22' north latitude and 71°-4'

east longitude, higher temperature 49°C during hotter season and 1°C during winter season. The average rainfall in district Multan is of 127 mm. The present survey was conducted on *Theileria* spp. infection and changes in hematological and biochemical values in *Theileria* infected small ruminants in Multan.

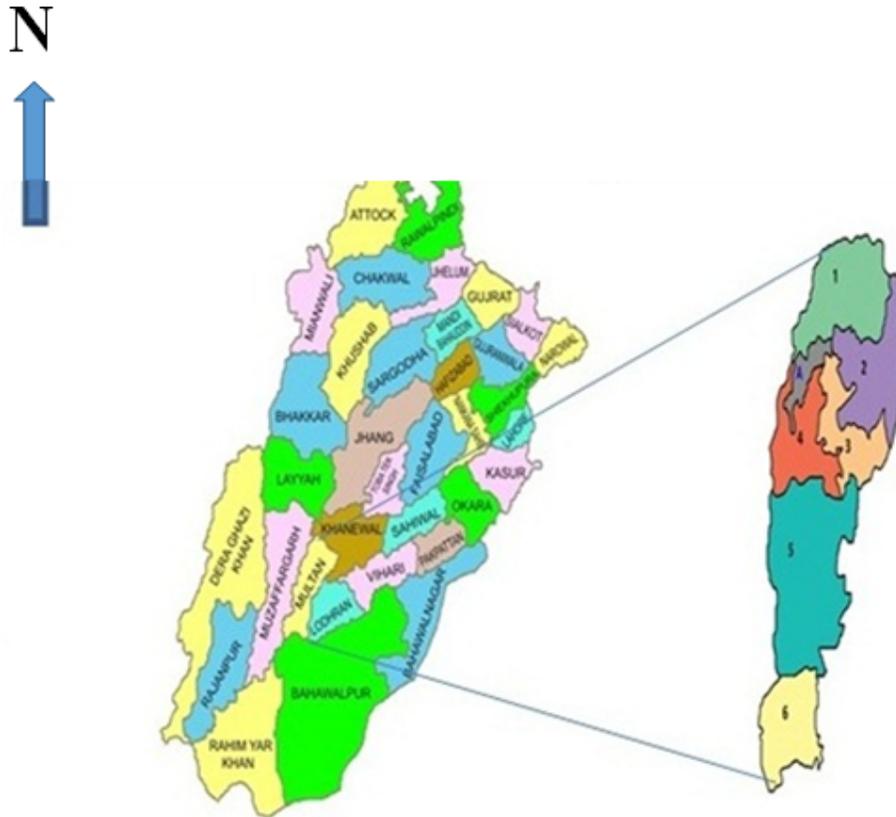


Figure-1. Study area of district Multan, Punjab, Pakistan.

Blood sampling

200 blood samples (sheep = 144, goats = 56) were collected from apparently healthy small ruminants from selected herds located at different places during 2013. 10 milliliter (ml) blood was collected from sampled animals by puncturing jugular vein with sterilized syringe. 5 ml collected blood was poured in eppendorf having few drops of EDTA for extraction of DNA for PCR amplification; while other 5 ml collected blood was used for serum collection in order to determine hematobiochemical analysis. The collected blood samples were properly labeled including date, location and characteristics of animals as well as herds.

Microscopic examination

Thin blood smears were prepared in the field, then air dried and fixed in methanol (absolute) for 1 minute in the field. The blood smears were stained with Giemsa (5%) for 30 minutes in the laboratory and

enquired *Theileria* spp. under immersion oil lens ($\times 1000$). 250 microscopic fields were surveyed to identify the infected RBCs in order to assess the parasitemia ratio in the infected animals Jalali *et al.* (2014).

PCR amplification

Inorganic method was used for DNA extraction Shaikh *et al.* (2015). The quality of extracted DNA was evaluated by spectrophotometer analysis at 260/280 nm density constant and gel electrophoresis. The extracted DNA was used for PCR amplification. The primers set 989 F; 5'-AGTTTCTGACCTATCAG-3' and 990 R; 5'-TTGCCTAAACTTCCTTG-3' (Penicon) were used for amplification of 1098 bp portion of the *ssurRNA* gene of genus *Theileria*. The final 50 μ l PCR mixture contained of 5 μ l (1 ng μ l⁻¹) of template DNA, 5 μ l of 10 X PCR buffer (100 mM Tris-HCl (pH 9) 500 mM KCl, 1% Triton X-100), 5 μ l of 50 mM MgCl₂, 6 μ l of dNTPs, 4 μ l of each primer (μ M) (Penicon) at a concentration of 10 pmol/ μ l, 2



U of Taq DNA polymerase (Vivintas) and 20.5 µl of PCR water. PCR amplification was done using a programmable thermal cycler (BIORAD). The cyclic conditions were at 94 °C for 5 min (initial denaturing step), followed by 35 cycles each at 94 °C for 1 min (denaturing step), at 60 °C for 1 min (annealing step) and 72 °C for 1 min (extension step) with a final extension step at 72 °C for 7 min. PCR amplified products were separated on 1.5% solidified agarose gel in TBE buffer and visualized by using ethidium bromide and UV-illuminator. The 100-1500 bp ladder (Vivantus) was used as DNA marker. Positive control genomic DNA of *Theileria* was provided by Professor UrikeSeitzer (VIIRC, Borstel, Germany).

Haematological and Serum biochemical studies

The haematological values such as RBCs, WBCs, HB concentration, PCV, MCV, MCH and MCHC values were recorded of infected and healthy small ruminants. The sera of infected and healthy animals were analyzed for the measuring of total protein, albumin, globulins, cholesterol, calcitonin and urea level using commercial test kits.

Statistical analysis

MiniTab (Version 16) was used for statistical analysis. One way Analysis of Variance (ANOVA) was

used to compare and determine significant difference of hematological and biochemical parameters of laboratory obtained values between healthy and infected animals with theileriosis. All values were expressed as mean and standard error (SE). Fisher's exact test and Pearson's Chi square test was used to find association between different variables of (animals and herds) and ovine theileriosis. A $p < 0.05$ value was considered as statistically significant

RESULTS AND DISCUSSIONS

Blood smear examination and clinical symptoms based diagnosis are useful in acute cases but meager in subclinical cases of theileriosis. As opponent to these methods, molecular assays are better for detection of piroplasms in carrier animals. PCR amplification method is more specific and sensitive than microscopy for diagnosis of *Theileria* piroplasms. A total of 12.5% and 39.5% blood samples found *Theileria* infected by microscopy and PCR respectively during present study. The details of sampling area, number of samples collected and *Theileria* positive are listed in (Table-1). The higher prevalence of *Theileria* spp. was identified in Basti Lotahar (66.7%) while the lowest in Basti Allah dad (20%) based on PCR amplification. Chi square results indicated significant correlation of ovine theileriosis between different sampling sites ($p < 0.05$).

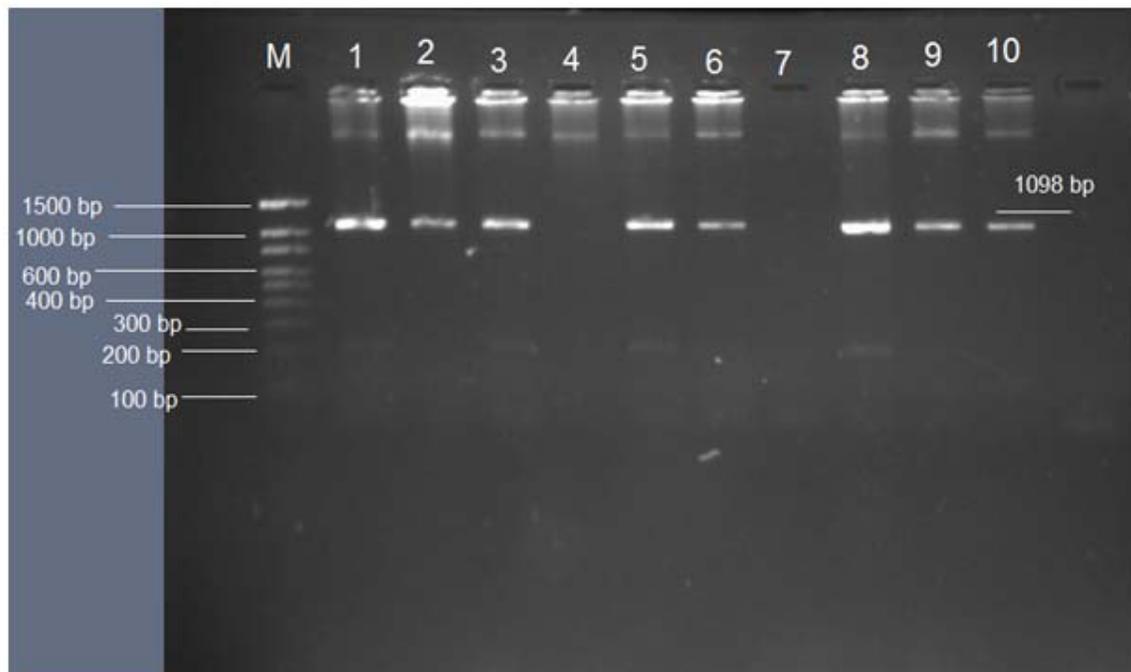


Figure-2. Agarose gel electrophoresis of amplified PCR products obtained from *Theileria* species genomic DNA using *Theileria* specific primers. Lane M. DNA marker of 100, 1500bp; Lane 1. *Theileria* species DNA positive control; Lane 4. *Theileria* species Negative control (Distilled water); Lane 2,3,5,6,8,9,10. *Theileria* species positive samples; Lane 7. *Theileria* species negative samples.

**Table-1.** Microscopic examination of thin blood smears and PCR amplification results of sheep and goats from Multan, Pakistan.

Area	No. of samples		Test			
		Microscopic examination		PCR Examination		P*value
		Positive	%	Positive	%	
BastiMongwad	25	3	12	7	28	
BastiLotahar	30	5	16.7	20	66.7	
BastiAladad	25	3	12	5	20	
BastiShhadanlund	30	4	13.3	10	33.3	
MazaTatypur	50	8	16	27	54	0.000 ^{b***}
MozaKarnalpur	40	2	5	10	25	
Total	200	25	12.5	79	39.5	0.000 ^{a***}

a = Fisher's exact test b = Chi square test;

P < 0.01 = Significant (**); P < 0.001 = highly significant (***)

The hematobiochemical profile is a significant tool to distinguish between healthy and diseased animals in veterinary studies. The results of present study shown ovine *Theileria* infection led to alterations in some hematobiochemical parameters. The significant ($p < 0.05$) decrease in RBCs counts, Hb contents and PCV values was found between normal and *Theileria* infected animals. Lower RBCs count, Hb contents and PCV had been found in *Theileria* infected small ruminants which are in accordance to present study Razavi *et al.* (2011), Nazifi *et al.* (2011) and Bell *et al.* (2004). Cattle infected with *Theileria annulata* also shown decline in RBCs count, Hb contents and PCV values Al-Sultan *et al.* (1999) and Karawan (2007). The lower hematological values in animals with clinical signs suggest a benign microcytic and hyperchromic anemia. Extensive hemorrhages, abdominal ulcers and insistence of parasitic stages in erythrocyte lead to lower hematological values Geerts *et al.* (2001). This stress might attribute erythrocytic fragility because of membrane lysis and lower haemoglobin concentration in infected animals Mehta *et al.* (1998) and Rayulu *et al.* (1995). The decline of RBCs count, Hb contents and PCV values might endorsed the deficit of erythrocytes resulted by invasion of macrophages in lymph nodes, spleen and other organs of reticuloendothelial system. The oxidative stress increased in infected animals cannot be abridged by the antioxidant enzymatic activity Grewal *et al.* (2005). The decline in RBCs count and HB contents was due to higher parasitaemia level in the infected small ruminants Omer *et al.* (2002) and led to severe anemia in the *Theileria* spp. infected animals Watarai *et al.* (1995). Surface membrane changes, increase the osmotic fragility and variations of

glycolipids of red blood cells membrane and oxidative damages resulted in anemia in diseased animals Yagi *et al.* (2002) and Shiono *et al.* (2004). Destruction of RBCs counts during anemia in infected animals might be due to fastening of autoantibody to infected RBCs which later phagocytized Ahmed (2002) or due to cytokine tumor necrosis factor (TNF α) which is effective inducer of fever and might play a role in development of anemia Campbell *et al.* (1995). Removal of piroplasms from infected erythrocytes by macrophages could be the source of anemia in the *Theileria* infected animals Al-Amery *et al.* (2002). The reduction in RBCs count might be due to erythropagocytosis during theileriosis and resulted in higher oxygen radicals caused anemia Shiono *et al.* (2004).

During present study, lower values of MCV found between healthy and *Theileria* spp. infected animals. Similar trend of significantly ($p < 0.05$) higher MCV value was found during malignant ovine and bovine theileriosis Singh *et al.* (2001). Concerning the erythrocyte indices with parasitemia rates a significant depletion in MCV value [17]. Significant ($p < 0.05$) decreases were found in MCH and MCHC values in the animal infected with ovine theileriosis. Hematological analysis revealed significant increase ($p < 0.05$) in WBCs count in infected small ruminants compared with healthy animals. The higher erythrocytes had been found in sheep infected with *Theileria* spp. compared with control group Al-Obaidi *et al.* (2004) and Yasini *et al.* (2012) but contradicts to that of [35] who showed non-significant leukocytosis in animals experimentally infected with *Theileria* spp. infection which might be due to proliferation of lymphocytes as defensive retort to attacking parasite.

**Table-2.** Mean values of hematological parameters in affected and apparently healthy small ruminants.

S. No.	Parameters	Normal Group (n=100)	Infected Group (n=50)
1	WBC × 10 ³ / μL	31.8±1.55	38.1±1.65***
2	RBC × 10 ⁶ / μL	6.31±0.12	5.9±0.33
3	HGB g/dL	7.4±0.12	7.1±0.12***
4	PCV%	26.8±0.60	16.4±0.98***
5	MCV fL	33.6±0.97	33.2±0.81
6	MCH pg	18.2±1.7	12.3±0.91***
7	MCHC g/dL	40.8±2.9	35.5±2.30***

*** Statistically significant

Table-3. Mean values of serum biochemical parameters in affected and apparently healthy small ruminants.

S. No.	Parameters	Normal Group (n=100)	Infected Group (n=50)
1	Total protein g/dL	36.3±1.00	37.5±1.60
2	Albumin g/dL	2.77±0.03	2.5±0.03***
3	Globulin g/dL	4.6±0.17	4.3±0.07
4	Cholesterol mg/Dl	97.0±3.50	98.9±2.26
5	Calcitonin mg/dL	0.76±0.03	0.78±0.01
6	Urea mg/dL	36.3±1.03	37.5±1.60

*** Statistically significant

During current investigation, serum total protein and globulins concentration showed non-significant reduction ($p > 0.05$) while albumin concentration showed significant decline in animals with *Theileria* spp. infection compared normal animals ($p < 0.05$). Similar trends of hypoproteinaemia and hypoalbuminaemia was found in small ruminants diseased with theileriosis Sandhu *et al.* (1998). The significant decrease in albumin level attributed reduced synthesis of proteins because of liver impairment due to *Theileria* spp. Infection Singh *et al.* (2001). Moreover lower serum protein concentration could be ascribed due to extravascular amassing of proteinaceous fluids, resulting from diseased lymph nodes Col *et al.* (2007). During theileriosis lower serum protein level could be due to shortage of dietary intake, diarrhea and lower production due parasitic infection on the liver Al-Obaidi *et al.* (2004). The major sites of synthesis of plasma protein are liver but severe tissue damages in the liver occurs during bovine theileriosis [38]. The decreased serum protein level in animals naturally infected with *Theileria* spp. infection was due to hypoalbuminaemia and hypoglobulinaemia because of liver damage Singh *et al.* (2001) and Watarai *et al.* (1995). The results of current study revealed non-significant rise in urea level in *Theileria* spp. infected animals might be endorsed to kidney damage Watarai *et al.* (1995). The higher urea level in *Theileria* spp. infected animals endorsed to histopathological variations resulted in the renal

parenchyma due to piroplasmiasis and shown strong correlation with the level of parasitemia. Non-significant rise in cholesterol level found in animals infected with theileriosis during present study was in accordance to significantly higher level of cholesterol reported in cattle infected with theileriosis Shahnawaz *et al.* (2011), but contradicts to Baghshani *et al.* (2011). Who reported non-significant increase of cholesterol levels in small ruminants infected with theileriosis. The higher cholesterol level endorsed to liver impairment due to parallel higher fats contents and lower sugar and protein level Yadav *et al.* (1986).

CONCLUSIONS

This is the first preliminary study of *Theileria* infection in sheep and goats of Multan, southern Punjab, Pakistan which has a deleterious influence health of small ruminants. The current study epitomizes that ovine theileriosis significantly affect the hematological and biochemical parameters in the infected animals that are in the early stage of disease without any appearance of clinical symptoms. Further research is requisite to trace the variations in haematobiochemical parameters due to *Theileria* spp. infection.

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the data: A Sohail, Contributed reagents/ materials/ analysis tools: Farhatullah, I H Khalil & S M A Shah Wrote the paper: A Sohail.

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