

## A report on the molecular detection and seasonal prevalence of *Trypanosoma brucei* in Dromedary Camels from Dera Ghazi Khan District in Southern Punjab (Pakistan)

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**Abstract.** The present study was designed for molecular detection of *Trypanosoma brucei* through PCR, by using kinetoplast DNA (kDNA) maxicircle primers, on seasonal basis and to demonstrate the effect of this parasite on complete blood count and selected parameters of serum biochemistry in camels from Southern Punjab (Pakistan). A total of 291 camel blood samples (61 male, 230 females) were collected from Dera Ghazi Khan District in Pakistan during March 2012 till February 2013 for *Trypanosoma brucei* detection by blood smear screening, micro hemato centrifugation and Polymerase chain reaction techniques. Twenty eight out of 291 blood samples (9.62%) produced a 164 bp DNA fragment specific for *T. brucei*. Only 6 blood samples (2.06%) were found parasite positive by microscopic examination and 13 (4.46%) were positive for microhematocrit centrifugation technique. Seasonal PCR based prevalence of trypanosomiasis was 6.9%, 13.7%, 9.7% and 8.1% during spring, summer, autumn and winter seasons respectively. *T. brucei* prevalence was not restricted to a particular age group or gender of the studied animals ( $P > 0.05$ ). A significant increase in WBC ( $P = 0.001$ ), neutrophils ( $P = 0.004$ ), ALT ( $P = 0.028$ ) and decreased RBC ( $P < 0.000$ ), hemoglobin ( $P < 0.000$ ) and packed cell volume ( $P < 0.000$ ) were detected in parasite positive as compared to the parasite negative blood samples. In conclusion, PCR is a more reliable and sensitive technique than conventional microscopic blood screening and microhematocrit centrifugation for the detection of *T. brucei* in camel blood. We recommend the use of PCR for the effective prophylactic detection of *T. brucei* in livestock in order to reduce economic losses.

### INTRODUCTION

Livestock contributes 53.2% in agriculture sector of Pakistan and accounts for 11.4 % of the Gross domestic product (GDP). This contribution is higher than from the crop sector (47.4% in agriculture and 10.3% in total GDP) that is major earning sector in Pakistan (Aslam *et al.*, 2010). Camels are raised in this country for draught, farming, transportation,

meat, milk and for sports in rural as well as in urban areas (Bhutto *et al.*, 2010).

Trypanosomiasis is a blood borne pathological condition and it is among the major constraints for the development of livestock industry in developing countries like Pakistan. In equines and camels, trypanosomiasis is found as acute or chronic infection and it is caused by a Protozoan parasite, *Trypanosoma* sp. (Konnai *et al.*,

2009). Tsetse fly act as vector for the transmission of this parasites (Shah *et al.*, 2004; Gutierrez *et al.*, 2005). Disease symptom includes intermittent fever, loss of appetite, lacrimation of eyes, petechial hemorrhages in conjunctiva of eyes, anemia, edema of limbs and genitalia, enlarged lymph nodes, abortion, decreased fertility and loss of body weight leading to early death in acute forms of disease (Ngaira *et al.*, 2002). While nervous signs with emaciation and death are observed in chronic forms of trypanosomiasis (Clausen *et al.*, 2003).

Limited information is available regarding the status of trypanosomiasis in Pakistan. So the present study was design to report a specific, reliable and sensitive molecular tool, the polymerase chain reaction (PCR), for the detection of *T. brucei* in blood samples of camel from Southern Punjab. Furthermore, the present study provided a baseline data regarding *T. brucei* prevalence and risk factors involved in the spread of trypanosomiasis and we have also demonstrated the effect of trypanosomiasis on the complete blood count and selected parameters of serum biochemistry parameters in camels.

## MATERIALS AND METHODS

### Sample and Data Collection

Blood samples, from Jugular vein, of 291 camels were randomly collected from Dera Ghazi Khan (N = 291) district of Punjab in 2013. A questionnaire was filled at the sampling site in order to determine whether age and gender of the subjects have any association with the prevalence of the parasite. All the animal handling procedures and experimental protocols were approved by the ethical committee of Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University Multan, Pakistan (Approval No. EB/IPAB1788/2013).

### DNA Extraction

Inorganic method of DNA extraction was used following Razzaq *et al.* (2015). The quality of the DNA extracted for purity and

integrity was assessed with optical density counts at 260/280.

### Polymerase Chain Reaction (PCR)

A set of oligonucleotide primers, TBR 1, 5'GAATATTAACAATGCGCAG 3' and TBR2 5'CCATTTATTAGCTTTGTTGC 3', was used to amplify the maxicircle kinetoplast DNA (kDNA) of *Trypanosoma brucei* as previously described by Li *et al.* (2007).

PCR was performed in a final reaction volume of 25 ml containing 1X Taq Buffer, 0.2 mM dNTP mixture, 1.5 mM Magnesium chloride, 2.5 U/μl Taq Polymerase (Fermentas, UK), 4 μM of each primer, 2 μl of DNA template and DNase free de-ionized water. For the negative control, water was used instead of DNA, whereas DNA extracted from clinically confirmed positive sample was used as positive control.

DNA amplification was carried out in a DNA thermal cycler (Gene Amp<sup>®</sup> PCR system 2700 Applied Biosystems Inc., UK). The thermo-profile used by Ijaz *et al.* (1998) was modified for the present study with an initial denaturation carried out at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1 min, elongation at 72°C for 1 min and final extension was carried out at 72°C for 10 min. PCR products were held at 4°C until separated by electrophoresis on a 2.5% agarose gel and visualized under a UV Trans illuminator (Biostep, Germany).

### Blood smear formation

Blood smears were prepared for the detection of *T. brucei* in blood. A fresh blood drop was placed on a clean slide and dragged to form a smear with the help of a second glass slide inclined at an angle of 45° to the first one. The smears were fixed in absolute methanol and stained with Giemsa. Slides were observed under high power (100X) of microscope (Nikon, USA) by using immersion oil following Shahnawaz *et al.* (2011).

### Hematocentrifugation

Capillary tubes were centrifuged at 1020 g for 5 min and the presence of trypanosomes was detected by observation of parasite

motion just above the buffy coat. In a sample where trypanosomes were detected, the microhematocrit tube was cut just below the buffy coat to include 1 mm of the erythrocyte layer, and the contents including about 1 cm of plasma were transferred to a glass slide and covered with a cover slip. The wet smear was observed for trypanosomes under the microscope with reduced illumination following Biryomumaisho *et al.* (2009).

### Hematological and Serological Analysis

Various hematological [Red blood cells (RBC), White blood cells (WBC), Hemoglobin (Hb), Packed cell volume (PCV), Mean cell volume (MCV), Mean cell hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Neutrophils (NUT) and Lymphocytes (LYM)] and serum biochemical parameters [Total proteins (TP), Creatinine, Alanine Transaminase (ALT), Aspartate transaminase (AST) and Triglycerides] were determined in *T. brucei* positive and negative blood samples by using Metertek SP-8SO spectrophotometer (Korea).

### Statistical Analysis

All the data is presented as Mean  $\pm$  Standard Error. Statistical package Minitab (version 16, USA) was used for the statistical analysis of the results. Chi-square test was used to determine the effect of season on parasite prevalence. Animals were divided into two age groups, animals up to 5 years (young) and animals more than 5 years old (mature). Association between the presence of *T. brucei* and various risk factors, i.e. gender

and age of animal was evaluated by contingency table analysis using the Fisher's exact test (for 2 x 2 tables). 2 sample t-test was calculated to compare various studied hematological and serum biochemistry parameters between *T. brucei* positive and negative blood samples.

## RESULTS

### Prevalence of *Trypanosoma sp.*

PCR results revealed that 28 out of 291 (9.6%) blood samples amplified a 164-bp amplicon specific for *T. brucei*. Variation in *T. brucei* prevalence was observed when parasite prevalence was correlated with four seasons of the study year but the difference in parasite prevalence did not reach statistical significance ( $P = 0.53$ ). Minimum parasites were detected in blood samples collected during spring (6.0%) while maximum (13.7%) *T. brucei* were detected during summer season of 2013 (Table 1).

### Sensitivity of methods for *Trypanosoma brucei* detection

Six blood samples (2.1%) were found *Trypanosoma sp.* positive by microscopic examination (Fig. 1) while 13 (4.5%) camel blood samples were confirmed to be positive for *T. brucei* by Microhematocrit centrifugation. On the other hand, 28 (9.6%) blood samples were found *T. brucei* positive upon PCR amplification including those samples found *Trypanosoma sp.* positive by blood smear screening and by

Table 1. Total number of camel blood samples collected (N) during the four sampling seasons from Dera Ghazi Khan District during 2013. Prevalence of *Trypanosoma sp.* is given in parenthesis. P value indicates the probability of chi-square test

Sampling season	N	<i>Trypanosoma sp.</i> +ive	<i>Trypanosoma sp.</i> -ive	P -value
Spring	72	5 (6.9%)	67 (93.1%)	0.53
Summer	73	10 (13.7%)	63 (86.3%)	
Autumn	72	7 (9.7%)	65 (91.3%)	
Winter	74	6 (8.1%)	68 (91.9%)	
Grand Total	291	28 (9.6%)	263 (91.4%)	

P > 0.05 = Non significant

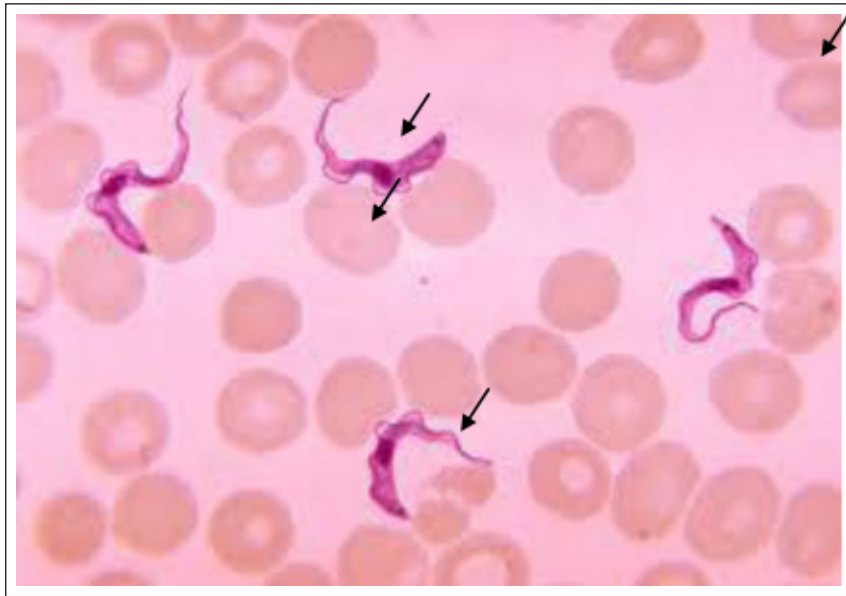


Figure 1. Photomicrograph of *Trypanosoma* sp. (Arrow) in a Giemsa stained blood smear from a dromedary camel. Magnification 100X.

microhematocrit centrifugation. This comparison of various techniques for the detection of parasite revealed that PCR is more sensitive and reliable tool than the conventional methods.

#### **Analysis of risk factors associated with trypanosomiasis**

Age and gender were correlated with the prevalence of *T. brucei*. Parasite was detected more frequently in males ( $P = 0.32$ ) than females and its prevalence was higher in camels older than 5 years of age than the younger ones ( $P = 0.09$ ) but the difference in prevalence, in both cases, did not reach statistical significance (Table 2). Analysis of results indicated that both the studied parameters had no association with the incidence of parasite (Table 2).

#### **Analysis of hematobiochemical parameters**

Analysis of the complete blood count results revealed *T. brucei* positive animals had significantly lower red blood count ( $P < 0.001$ ), hemoglobin concentration ( $P < 0.001$ ), packed cell volume ( $P < 0.001$ ) and lymphocytes (%) ( $P = 0.003$ ) than *T. brucei* negative camels indicating that the parasite

affects the red blood cell number in camels (Table 3). In parasite positive camels, number of white blood cells ( $P < 0.001$ ) and neutrophils ( $P = 0.004$ ) was significantly higher than *T. brucei* negative camels indicating an ongoing infection in these animals. While all other studied parameters varied non significantly between the two groups (Table 3). Among the studied serum biochemical parameters, Alanine Transaminase (ALT) concentrations were significantly higher ( $P = 0.03$ ) in parasite positive camels, indicating abnormal liver functioning in these animals (Table 3).

#### **DISCUSSION**

Parasitic diseases are the major limitations to the livestock industry as they globally affect human health, trade and economy (Razzaq *et al.*, 2015). Trypanosomiasis is reported in Africa, Europe, United States and Asia causing economic loss in these areas (Konnai *et al.*, 2009).

Trypanosomiasis among the widely distributed livestock associated pathological conditions in Asia and Africa. It is commonly reported from Egypt, Sudan, Somalia, Saudi

Table 2. Association between parasite prevalence in camel blood samples collected during four seasons in 2013 from Dera Ghazi Khan District and the studied parameters describing the animal characters. Prevalence of *Trypanosoma* sp. is given in parenthesis. Probability of Fisher's exact test (P-value) is mentioned for each parameter

Parameters		N	<i>Trypanosoma</i> sp. +ive	<i>Trypanosoma</i> sp. -ive	P-value
Sex	Male	61	8 (13.1%)	53 (86.9%)	0.33
	Female	230	20 (8.7%)	210 (91.3%)	
	All	291	28 (9.6%)	263 (90.4%)	
Age	> 5 Year	92	13 (14.1%)	79 (85.9%)	0.09
	< 5 Year	199	15 (7.5%)	184 (92.5%)	
	All	291	28 (9.62%)	263 (90.38%)	

P > 0.05 = Non significant

Table 3. Comparison of various hematobiochemical parameters between *Trypanosoma* sp. positive and negative camel samples from Dera Ghazi Khan District. Data is expressed as Mean  $\pm$  Standard Error of Mean. P-Value indicates the probability of 2 sample t-test

Parameters	<i>Trypanosoma</i> sp. positive samples (N = 28)	<i>Trypanosoma</i> sp. negative samples (N = 261)	P-value
RBCs ( $\times 10^6$ /ul)	6.05 $\pm$ 0.22	7.22 $\pm$ 0.08	0.000***
WBCs ( $\times 10^3$ /ul)	20.28 $\pm$ 1.2	15.64 $\pm$ 0.30	0.001***
Hb (gm/dl)	9.01 $\pm$ 0.29	10.63 $\pm$ 0.10	0.000***
PCV (%)	24.18 $\pm$ 1.0	31.6 $\pm$ 0.69	0.000***
MCV (fl)	41.3 $\pm$ 2.3	45.2 $\pm$ 1.1	0.129
MCH (pg)	15.38 $\pm$ 0.74	15.14 $\pm$ 0.20	0.759
MCHC (gm/dl)	38.21 $\pm$ 1.4	36.1 $\pm$ 0.52	0.168
NUT ( $\times 10^3$ /ul)	12.53 $\pm$ 1.2	8.6 $\pm$ 0.26	0.004**
LYM ( $\times 10^3$ /ul)	5.81 $\pm$ 0.45	6.43 $\pm$ 0.24	0.229
NUT (%)	60.2 $\pm$ 3.2	54.9 $\pm$ 1.1	0.119
LYM (%)	28.2 $\pm$ 2.6	37.3 $\pm$ 1.1	0.003**
Total Protein (gm/dl)	7.15 $\pm$ 0.19	7.55 $\pm$ 0.10	0.070
Creatinine (mg/dl)	1.50 $\pm$ 0.09	1.51 $\pm$ 0.03	0.869
ALT (U/L)	31.1 $\pm$ 4.2	21.1 $\pm$ 0.71	0.03*
AST (U/L)	74.3 $\pm$ 5.4	75.4 $\pm$ 1.7	0.846
Triglycerides (mg/dl)	38.7 $\pm$ 2.5	38.0 $\pm$ 0.87	0.783

RBC= Red blood cells, WBC= White blood cells, Hb= Hemoglobin, PCV= Packed cell volume, MCV= Mean cell volume, MCH= Mean cell hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, NUT= Neutrophils, LYM= Lymphocytes, ALT= Alanine Transaminase, AST= Aspartate transaminase.

P > 0.05 = Non Significant, P < 0.05 \* = Least Significant, P < 0.02 \*\* = Significant, P < 0.001 \*\*\* = Highly Significant.

Arabia, Iran and India. It has also been reported from Nigeria, Ethiopia, Kenya, Jordan and Iraq (Ouhelli & Dakkak 1987; Ijaz *et al.*, 1998; Holland *et al.*, 2001; Masiga *et al.*, 2001; Clausen *et al.*, 2003; Li *et al.*, 2007). There are also few reports from Southern Punjab, in Pakistan, regarding the prevalence of *T. evansi* and *T. brucei* in cattle and equines (Khan, 1986; Waheed *et al.*, 1998; Nasir *et al.*, 1999; Hasan *et al.*, 2006; Aslam *et al.*, 2010; Shahzad *et al.*, 2012) but no comprehensive data is available in literature regarding the PCR based prevalence of *T. brucei* in camels from Pakistan. Shah *et al.* (2004) had reported that 102 camel blood samples, collected from Sindh Province in Pakistan, were infected with *Trypanosoma* sp. but only 14 (13.72%) were confirmed to be positive for *Trypanosoma evansi*. In another recent study, Bhutto *et al.* (2010) has reported 11.25% prevalence of *Trypanosoma evansi* in randomly collected 240 camel blood samples from various districts of Sindh Province. Murtaza *et al.* (2006) has reported that five (3.3%) and six (4%) camels out of 150 dromedary camels from Punjab were positive for *Trypanosoma evansi* for parasitological and serological examination, respectively. The present study reports the presence of *Trypanosoma brucei* in 9.6% of the blood samples collected from randomly selected dromedary camels from Southern Punjab (Table 1). Our reported prevalence of the parasite is higher than that reported by Murtaza *et al.* (2006) from Punjab but this type of discrepancy can be attributed to variations in the ecology of the study areas and seasons of the year when the blood sampling was conducted as it directly effects the distribution of biting flies responsible for the mechanical transmission of *T. brucei*. Another important factor, that is a major reason for the difference in parasite prevalence between the two studies, is the different techniques of parasite detection as we are reporting PCR based detection, while Murtaza *et al.* (2006) has detected the parasite by blood smear scanning and ELISA.

Several methods has been developed for the detection of *Trypanosoma* sp. infection including microscopy, card agglutination test Gutierrez *et al.* (2000); microhaematocrit

centrifugation technique (Biryomumaisho *et al.*, 2009); enzyme linked immunosorbent assay Indrakamhang *et al.* (1996); DNA hybridization Viseshakul *et al.* (1990) and polymerase chain reaction Li *et al.* (2007) and PCR has been proved to be the most sensitive and reliable tool for the detection of *Trypanosoma* sp. In the present study, we have reported similar observations as *T. brucei* was detected in 2.1, 4.5 and 9.6% blood samples respectively by microscopic examination, microhematocrit centrifugation and PCR. The sample that were found parasite positive by smear screening and microhematocrit centrifugation were also positive for *Trypanosoma brucei* during their PCR detection.

Data regarding age and gender of the animals indicated that male and adult animals were more infected with *T. brucei* than the female and young ones but this association was statistically non-significant (Table 3). Our findings are in agreement with the results of Bogale *et al.* (2012) who had reported the higher prevalence of *Trypanosoma evansi* in male than female camels from Ethiopia. They have also reported higher infection adults than the young ones but these results did not reached statistical significance. The possible reason why young were less infected than adults could be due to the fact that pastoralists keep them in the residence area and they do not go to distant areas where the fly burden is high (Kassa *et al.*, 2011). Our results are also in agreement with Bhutto *et al.* (2010) as they have reported that *Trypanosoma evansi* prevalence was higher in male than female camels although this difference was not statistically significant. This could be due to the fact that male camels travel from one place to another place to provide transportation service more than female camels, so that they have a higher probability of acquiring an infection. Frequent travel can also compromise their immune response to infection due to the stress of fatigue (Kassa *et al.*, 2011).

Various haematological parameters were also compared between *Trypanosoma* sp. positive (N = 28) and negative (N = 163) blood samples. Our results indicated that presence of the parasite has significantly affected the

red and white blood cell count (Table 3). Red blood cells and related parameters were significantly lower in *T. brucei* Positive than negative blood samples while number of white blood cells was higher in parasite positive samples indicating infection caused by *Trypanosoma* sp. These results are in agreement with those reported by Padmaja (2012) who had reported a significant ( $P < 0.01$ ) increase in temperature and decrease in red blood cell and hemoglobin while a significant increase ( $P < 0.05$ ) in lymphocytes, neutrophils and eosinophils in *T. brucei* positive camel blood samples.

In conclusion, we have reported that PCR is more sensitive and reliable tool for the detection of *T. brucei* in camel blood samples than blood smear screening and microhematocrit centrifugation. *T. brucei* was detected in all four seasons of the sampling year with higher prevalence in summer. Presence of *T. brucei* has significantly affected the blood chemistry that may lead to production losses. We recommend the PCR based screening of this parasite in Pakistan as trypanosomiasis is common in Pakistan, in order to prevent economic loss and improve their productivity.

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