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Molecular Detection and Seasonal Prevalence of *Trypanosoma brucei* and its Effect on Hematobiochemical Parameters in Donkeys from Dera Ghazi Khan District in Southern Punjab, Pakistan

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ABSTRACT

The present study was designed to report the prevalenceof *Trypanosoma brucei* in blood samples of donkeys (n=283) collected from Dera Ghazi Khan District in Punjab (Pakistan) through PCR amplification of their maxicircle kinetoplast DNA (kDNA) during four seasons in 2013 and to demonstrate the effect of this parasite on complete blood count (CBC) and selected parameters of serum. Out of 283 blood samples, 19 (6.71%) generated a 164 bp DNA fragment specific for *T. brucei*. Only 7 blood samples (2.5%) were found *Trypanosome* sp. positive through microscopic examination of blood smears while 13 (4.6%) were found positive by microhematocrit centrifugation technique. Parasite prevalence was 4.76% (3/65) in spring, 8.22% (6/73) in summer, 8.45% (6/71) in autumn and 5.41% (4/74) in winter, detected by PCR. Analysis of results revealed no significant association of *T. brucei* prevalence with age ($P > 0.05$) and gender ($P > 0.05$) of animals. Comparison of hematobiochemical profile revealed a significant increase in white blood cell count ($P = 0.002$), neutrophil count ($P < 0.001$), neutrophil [%] ($P < 0.001$) and ALT ($P = 0.019$) while decrease in red blood cell count ($P < 0.001$), hemoglobin ($P = 0.003$), packed cell volume ($P = 0.003$) lymphocytes ($P < 0.001$) and lymphocytes [%] ($P < 0.001$) in *T. brucei* positive animals as compared to the animals where parasite was not detected in blood through PCR. We concluded that PCR is more reliable and sensitive technique for the detection of *T. brucei* in blood samples from donkeys than blood smear screening and microhematocrit centrifugation techniques . It is recommended to be used for the detection of this parasite in order to minimize economic losses.

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Authors' Contribution
FI had designed the study and revised the manuscript. MH, ZS and RSS had collected the blood samples and conducted the lab experiments. MG and MA had carried out the hematological analysis. MA analyzed the data and prepared the manuscript.

Key words
***Trypanosoma* sp., blood smear screening, hematocrit centrifugation, PCR, Donkey.**

INTRODUCTION

Livestock contributes 53.2% in agriculture sector and it is about 11.4 % of the total gross domestic product (GDP) of Pakistan which is higher than the contributions from the crop sector (47.4% in agriculture and 10.3% in total GDP) (Aslam *et al*., 2010; Bhutto *et al.*, 2010). Equines (horses, donkeys and mules) are raised by man since long for draught and game purposes and are playing a major role in daily income generation of poor people they are usually kept for transportation of people, carriage of goods and agricultural purposes (Simenew *et al*., 2011).

Hemoparasitic diseases like trypanosomiasis are among the major constraints for the development of livestock industry especially in developing countries like Pakistan. In equines and camels, trypanosomiasis is found in acute or chronic forms and the infection is caused by protozoan parasites of the genus *Trypanosoma* (Urquhart *et al*., 1996; Konnai *et al*., 2009) transmitted mainly by Tsetse fly (Shah *et al.*, 2004; Gutierrez *et al.*, 2005) and the disease symptoms 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) and nervous signs with emaciation and death (in chronic forms) (Ngaira *et al.*, 2002; Clausen *et al.*, 2003).

A little information is available regarding the prevalence of *T. brucei* in Southern Punjab. Hence, the present study was designed to compare the sensitivity of various biological tools (blood smear screening, microhematocrit centrifugation and PCR) for the detection of *Trypanosoma* sp. in blood samples of donkeys from Southern Punjab. Furthermore, the present study provided a baseline data regarding *T. brucei* prevalence and risk factors associated with the parasite prevalence along with the effect of *T. brucei* on the complete blood count and selected parameters of serum in donkeys.

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MATERIALS AND METHODS

Sample and data collection

A total of 283 blood samples were randomly collected from jugular vein of donkeys from Dera Ghazi Khan (n=283) district in Punjab during the four seasons 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.

PCR amplification of kDNA

Inorganic method of DNA extraction was used following Razzaq *et al.* (2015). The quality of the extracted DNA for purity and integrity was assessed with optical density counts at 260/280nm by using a spectrophotometer.

A set of oligonucleotide primers,
TBR 1, 5´GAATATTAAACAATGCGCAG 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 µl containing 1X Taq Buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 2.5 U/µl Taq polymerase (Fermentas, UK), 4 µM of each primer, 2 µl of DNA template and DNase free de-ionized water. DNA extracted from the blood of donkeys having clinical symptoms of trypanosomiasis with *Trypanosoma* detected in blood by smear screening was used as positive control. For the negative control, water was used instead of DNA, whereas DNA extracted from clinically and microscopically confirmed *Trypanosoma* sp. positive sample was used as positive control.

DNA amplification was carried out in a DNA thermal cycler (Gene Amp® 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 at 94ºC for 10 min followed by 35 cycles of denaturation at 94ºC for 1min, annealing at 55ºC for 1min, elongation at 72ºC for 1 min and final extension was carried out at 72ºC for 10 min. PCR products were held at 4ºC until separated by electrophoresis on a 2.5% agarose gel and visualized under a UV Trans illuminator (Biostep, Germany).

Blood smear detection of Trypanosoma *sp.*

Blood smears were prepared for the detection of *Trypanosoma* sp. 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.

Hematocentrifugation technique of Trypanosoma *detection*

Capillary tubes were filled with blood and centrifuged at 1020 *g* for 5 min and the presence of trypanosomes was detected by observing the movement of parasite 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 the confirmation of trypanosomes under the microscope with reduced illumination following Biryomumaisho *et al.* (2009).

Hematological and biochemical analysis

Various hematological parameters such as 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), lymphocytes (LYM) and serum biochemical parameters [Total proteins (TP), creatinine, alanine transaminase (ALT), aspartate transaminase (AST) and triglycerides were analyzed by using spectrophotometer (Metertek SP-8SO, Korea) and compared between *Trypanosoma* sp. positive and negative blood samples following Shahnawaz *et al.* (2011).

Statistical analysis

All the data is presented as Mean ± 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 (adults). 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). Two 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 T. brucei

Analysis of results showed that 19 out of 283 (6.71%) blood samples were found parasite positive as they amplified a 164-bp amplicon specific for *Trypanosoma brucei*. Only 7 blood samples (2.5%) were found parasite positive through microscopic examination while 13 (4.6%) were parasite positive as detected by microhematocrit centrifugation technique. A variation in the prevalence of *T. brucei* in different seasons during present study was observed. Minimum parasite prevalence was detected in blood samples collected during spring (4.76%; 3/65) while maximum (8.45%; 6/71) *T. brucei* prevalence was detected during autumn season (Table I). Chi square test results revealed that difference in parasite prevalence over seasons did not reach the statistical significance (P>0.05) indicating that *T. brucei* was not restricted to a particular season and was present in the analyzed equines throughout the year (Table I).

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

Sampling season	N	*T. brucei* + ive	*T. brucei* –i ve	P –value
Spring	65	3 (4.76%)	62 (95.24%)	P > 0.05
Summer	73	6 (8.22%)	67 (91.78%)	
Autumn	71	6 (8.45%)	65 (91.55%)	
Winter	74	4 (5.41%)	70 (94.59%)	
Total	**283**	**19 (6.71%)**	**264 (93.29%)**	

P > 0.05 = Non significant

Methods for Trypanosoma *sp. detection*

Comparison of various techniques for the detection of trypanosomiasis in donkeys revealed that PCR is more sensitive and reliable tool as only 7 blood samples (2.5 %) were found parasite positive by microscopic examination while 13 (4.6%) were found *Trypanosoma* sp. positive by Microhematocrit centrifugation as compared to 19 (6.71%) *T. brucei* positive samples detected by PCR. All samples found positive for *T. brucei* by PCR were also positive by Microhematocrit centrifugation confirming the specificity of PCR.

Analysis of risk factors associated with the prevalence of T. brucei

Analysis of data revealed that characters of animals *i.e.* age and gender had no association (P > 0.05) with the prevalence of *T. brucei* (Table II). *T. brucei* was more frequent in males (P = 0.963) and in donkeys older than 5 years of age (P = 0.714) than females and younger animals but the difference in prevalence, in both cases, did not reach statistical significance (Table II).

Table II.- Association between *T. brucei* prevalence in blood samples of donkeys collected during four seasons in 2013 from Dera Ghazi Khan District with the studied parameters describing the animal characters. Prevalence of *T. brucei* is given in parenthesis. Probability of Fisher's exact test (P-value) is mentioned for each parameter.

Sampling season	N	*T. brucei* + ive	*T. brucei* –i ve	P –value
Sex				
Male	276	19 (6.88%)	257 (93.12%)	0.963
Female	07	0 (0%)	07 (100%)	
Total	**283**	**19 (6.71%)**	**264 (93.29%)**	
Age				
> 5 Year	35	3 (8.57%)	32 (91.43%)	0.714
< 5 Year	248	16 (6.45%)	232 (93.55%)	
Total	**283**	**19 (6.71%)**	**264 (93.29%)**	

P > 0.05 = Non significant

Analysis of hematobiochemical parameters

Analysis of the complete blood count results indicated that *T. brucei* affected animals had significantly lower red blood count (P<0.000), hemoglobin concentration (P<0.003), packed cell volume (P<0.003) and lymphocytes (P = 0.000) than *T. brucei* negative animals (Table III). In parasite positive donkeys, number of white blood cells (P<0.002) and neutrophils (P = 0.000) were significantly higher than *T. brucei* negative donkeys indicating an ongoing infection in these animals. While all other studied parameters varied non-significantly (P>0.05) when compared between the *T. brucei* positive and negative animals (Table III). Among the studied serum biochemical parameters, Alanine Transaminase (ALT) concentrations were significantly higher (P = 0.019) in parasite positive donkeys indicating anomalous liver performance in these animals (Table III).

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 is common in Egypt, Sudan, Somalia, Saudi Arabia, Iran and India and has also been reported from Nigeria, Ethiopia, Kenya,

Jordan and Iraq (Ouhelli and Dakkak, 1987; Ijaz *et al.,* 1998; Holland *et al*., 2001; Masiga and Nyangao, 2001; Clausen *et al*., 2003; Li *et al*., 2007). The aim of present study was to analyze the prevalence of *T. brucei* in blood samples of donkeys, on seasonal basis, from Dera Ghazi Khan District of Punjab and to demonstrate the effect of this parasite on hematobiochemical parameters.

Table III.- Comparison of various hematobiochemical parameters between *T. brucei* positive and negative samples of donkeys collected from Dera Ghazi Khan District. Data is expressed as Mean ± Standard Error of Mean. P-value indicates the probability of 2 sample t-test.

Parameters	*T. brucei* positive blood samples (n = 19)	*T. brucei* negative blood samples (n = 264)	P-value
RBCs (x10^6/ul)	3.77 ± 0.27	5.08 ± 0.13	0.000***
WBCs (x10^3/ul)	14.23 ± 0.64	11.88 ± 0.20	0.002***
Hb (gm/dl)	7.96 ± 0.36	9.24 ± 0.11	0.003***
PCV (%)	20.31 ± 1.4	25.26 ± 0.33	0.003***
MCV (fl)	54.37 ± 2.2	52.1 ± 0.62	0.327
MCH (pg)	22.59 ± 1.3	19.37± 0.35	0.028*
MCHC (gm/dl)	42.6 ± 3.1	37.53 ± 0.48	0.128
NUT (x 10^3/ul)	8.22 ± 0.78	4.48 ± 0.20	0.000***
LYM (x 10^3/ul)	4.56 ± 0.4	6.47 ± 0.14	0.000***
NUT (%)	57.6 ± 4.6	37.1 ± 1.5	0.000***
LYM (%)	33.1 ± 3.3	58.1 ± 0.9	0.000***
Total protein (gm/dl)	8.01 ± 0.32	7.88 ± 0.08	0.705
Creatinine (mg/dl)	1.34 ±0 .11	1.28 ± 0.03	0.552
ALT (U/L)	38.5 ± 3.0	30.56 ± 0.51	0.019**
AST (U/L)	225.2 ± 9.3	227.6 ± 2.9	0.802
Triglycerides (mg/dl)	42.2 ± 3.8	45.2 ± 1.2	0.458

P>0.05 non Significant; *P<0.05, least significant; **P<0.02, significant; ***P<0.001, highly significant.
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.

The prevalence of *T. evansi* and *T. brucei* in cattle, camels and equines has been reported from Pakistan (Khan, 1986; Nasir *et al.*, 1999; Shahzad *et al*., 2012) but there is no comprehensive data on the use of PCR for detecting the infection in donkeys. The *Trypanosoma* infections have been reported in cattle, camels and equines from the different areas of Pakistan (Waheed *et al.*, 1998; Hasan *et al.*, 2006; Muhammad *et al.*, 2007; Aslam, 2009; Aslam *et al.*, 2010). Nasir *et al.* (1999) reported 32.5% (14 out of 43) Jersey cows and 23% (11 out of 47) Friesian cows positive for trypanosomes in a herd at a livestock experimental station, Bhunikey, Kasur, Pakistan. In a recent study, Bhutto *et al*. (2010) has reported 11.25% prevalence of *Trypanosoma evansi* in randomly collected 240 camels from various districts of Sindh Province. Likewise, a study conducted by Ravindran *et al*. (2008) using PCR to examine blood samples from 61 camels and 44 donkeys in India exhibited a prevalence of (21/61) 34.40% and (3/44) 6.8%, respectively. 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* at parasitological and serological examination, respectively. The present study reports the presence of *Trypanosoma brucei* in 6.71% of the blood samples collected from randomly selected dromedary camels from Southern Punjab (Table I). Our reported prevalence of the parasite is less than that reported by Bhutto *et al*. (2010) and Nasir *et al*. (1999) but this type of discrepancy can be attributed to variations in the ecology of the study areas and seasons of the year when the studies were conducted which have a direct effect on the distribution of biting flies responsible for the mechanical transmission of *Trypanosoma* sp.

Several methods has been developed for the detection of *Trypanosoma* sp. infection including microscopy, card agglutination test Guterrez *et al*. (2000); microhaematocrit centrifugation technique (Biryomumaisho *et al*., 2009); enzyme linked immunosorbent assay Indrakamhang *et al*. (1996); DNA hybridization Viseshakul and Panyim (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. Formerly the primer designated against *T. brucei* (TBR) was used to find out the species of trypanosomes prevalent in the Lahore district (Muieed *et al*., 2010). This primer was considered equally good for detection of the *T. evansi* gene suggesting that *T. brucei* and *T. evansi* are closely interrelated and have conserved sequence at least between TBR1 and TBR2 (Ijaz *et al.,* 1998). In the present study, we have reported similar observations as *Trypanosoma* sp. was detected in 2.5, 4.6 and 6.71% blood samples respectively by microscopic examination, microhematocrit centrifugation and PCR. The samples that were found parasite positive by smear screening and microhematocrit centrifugation were also positive for *T. brucei* during their PCR detection indicating more specificity of PCR. Similar observations were made by Ravindran *et al*. (2008) who reported a higher prevalence of *T. evansi* in camels, dogs, and donkeys through PCR than with blood smear examination.

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 II). Our findings are in agreement with the results of Bogale *et al.* (2012) who had reported the higher prevalence of *T. 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 *T. 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 *T. brucei* positive (n = 19) and negative (n = 264) blood samples. Our results indicated that presence of the parasite has significantly affected the RBC and WBC count (Table 3). RBCs and related parameters were significantly lower in *T. brucei* positive than in negative blood samples while number of white blood cells was higher in parasite positive samples indicating infection caused by *T. brucei*. 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 RBC and Hb, while a significant increase ($P<0.05$) in lymphocytes, neutrophils and eosinophils in *Trypanosoma* sp. positive blood samples. . Our findings are also in agreement with the works of Chaudhary and Iqbal (2000) and Gutierrez *et al.* (2005).

CONCLUSIONS

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

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Statement of conflict of interest

Authors have declared no conflict of interest.

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