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 prevalence of 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. 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.

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.
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´GAATATTAACAAATGCAGCAG 3´ and TBR2 5´CCATTATTAGCTTTG 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 MgCl2, 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 thermoprofile used by Ijaz et al. (1998) was modified for the present study with an initial denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, 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 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.

<table>
<thead>
<tr>
<th>Sampling season</th>
<th>N</th>
<th>T. brucei +ive</th>
<th>T. brucei -ive</th>
<th>P–value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td>65</td>
<td>3 (4.76%)</td>
<td>62 (95.24%)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Summer</td>
<td>73</td>
<td>6 (8.22%)</td>
<td>67 (91.78%)</td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>71</td>
<td>6 (8.45%)</td>
<td>65 (91.55%)</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>74</td>
<td>4 (5.41%)</td>
<td>70 (94.59%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>283</td>
<td>19 (6.71%)</td>
<td>264 (93.29%)</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Sampling season</th>
<th>N</th>
<th>T. brucei +ive</th>
<th>T. brucei -ive</th>
<th>P–value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>276</td>
<td>19 (6.88%)</td>
<td>257 (93.12%)</td>
<td>0.963</td>
</tr>
<tr>
<td>Female</td>
<td>07</td>
<td>0 (0%)</td>
<td>07 (100%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>283</td>
<td>19 (6.71%)</td>
<td>264 (93.29%)</td>
<td></td>
</tr>
</tbody>
</table>

| Age             |     |                |               |         |
| > 5 Year        | 35  | 3 (8.57%)      | 32 (91.43%)   | 0.714   |
| < 5 Year        | 248 | 16 (6.45%)     | 222 (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 (Razzag 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 two sample t-test.

<table>
<thead>
<tr>
<th>Parameters</th>
<th><em>T. brucei</em> positive blood samples (n = 19)</th>
<th><em>T. brucei</em> negative blood samples (n = 264)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBCs (x10^6/ul)</td>
<td>3.77 ± 0.27</td>
<td>5.08 ± 0.13</td>
<td>0.000***</td>
</tr>
<tr>
<td>WBCs (x10^6/ul)</td>
<td>14.23 ± 0.64</td>
<td>11.88 ± 0.20</td>
<td>0.002***</td>
</tr>
<tr>
<td>Hb (gm/dl)</td>
<td>7.96 ± 0.36</td>
<td>9.24 ± 0.11</td>
<td>0.003***</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>20.31 ± 1.4</td>
<td>25.26 ± 0.33</td>
<td>0.003***</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>54.37 ± 2.2</td>
<td>52.1 ± 0.62</td>
<td>0.327</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>22.59 ± 1.3</td>
<td>19.37 ± 0.35</td>
<td>0.028*</td>
</tr>
<tr>
<td>MCHC (gm/dl)</td>
<td>42.6 ± 3.1</td>
<td>37.53 ± 0.48</td>
<td>0.128</td>
</tr>
<tr>
<td>NUT (%)</td>
<td>4.56 ± 0.4</td>
<td>6.47 ± 0.14</td>
<td>0.000***</td>
</tr>
<tr>
<td>LYM (%)</td>
<td>57.6 ± 4.6</td>
<td>37.1 ± 1.5</td>
<td>0.000***</td>
</tr>
<tr>
<td>Total protein (gm/dl)</td>
<td>8.01 ± 0.32</td>
<td>7.88 ± 0.08</td>
<td>0.705</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.34 ± 0.11</td>
<td>1.28 ± 0.03</td>
<td>0.552</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>38.5 ± 3.0</td>
<td>30.56 ± 0.51</td>
<td>0.019**</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>225.2 ± 9.3</td>
<td>227.0 ± 2.9</td>
<td>0.802</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>42.2 ± 3.8</td>
<td>45.2 ± 1.2</td>
<td>0.458</td>
</tr>
</tbody>
</table>

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 trypansomes 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 Gutierrez 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, microhaematocrit centrifugation and PCR. The samples that were found parasite positive by smear screening and microhaematocrit centrifugation and PCR 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 reach 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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