Molecular Identification of Trypanosomes and Their Effects on Hematological and Biochemical Parameters in Donkeys in Punjab, Pakistan

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Abstract

The study was designed to investigate the molecular identification and prevalence and of trypanosomiasis and its effects on hematological and biochemical parameters in donkeys. Blood samples were collected from 657 donkeys of three districts: Gujranwala, Gujrat, and Mandi Bahaudin in Punjab province of Pakistan. Prevalence of Trypanosoma was observed by microscopy of Giemsa’s stained blood smear; whereas, serum and hematological parameters were determined by serum biochemistry and hematologic analyzer, respectively. Multiplex PCR was used to differentiate the species of Trypanosoma in diseased animals. Out of 657 donkeys screened, 58 (8.83%) were detected positive for trypanosomiasis by microscopic examination. Gujranwala was found to have highest prevalence (11.58%) followed by Gujrat (8.23%) and Mandi Bahaudin (5.39%), respectively. Hemoglobin, red blood cells count, packed cell volume and mean corpuscular volume were significantly (P<0.05) lower in infected animals; whereas, total leucocyte count and lymphocyte count were significantly (P<0.05) higher in infected animals. Total protein, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase were significantly (P<0.05) increased in the infected animals. Macrocyte, microcyte, acanthocyte, dacrocyte and bizarre shaped red blood cells were observed in infected animals. Multiplex PCR showed that Trypanosoma evansi was the most prevalent species. © 2018 Friends Science Publishers

Keywords: Molecular identification; Trypanosoma; Prevalence; Hematological values; Serum values

Introduction

Livestock accounted for 58.3% of the agriculture sector and 11.4% of the overall GDP during 2016-2017. Among various livestock species, working donkeys are the source of income generation for poor communities, being kept for transportation of people, goods, and many agricultural purposes (Simenew et al., 2011). Donkey population is estimated to be 5.2 million (Anonymous, 2016) in Pakistan with a growth rate of 2.95 per annum in Pakistan (Hasnain and Usmani, 2006). A number of parasitic, bacterial and viral diseases affect the donkey’s health and sometimes cause mortality in these animals. Trypanosoma (T.) is the etiological agent of trypanosomiasis in donkeys. Not only it causes disease in donkeys, but also it affects human population in the world particularly in Africa and Latin America. A recent study has revealed that Trypanosoma can infect all the domesticated animals (Fatihu et al., 2009).

Trypanosomiasis (Surra) is a chronic infection in the equine. The main species of trypanosomes that cause trypanosomiasis in animals are T. congolense, T. vivax and T. evansi (Abenga et al., 2002). They are transmitted biologically, but can also be transmitted through the mechanical means. The genus Glossina is the main vector for biological transmission (Clausen et al., 2003). In this disease, animals show intermittent fever, anorexia, severe weight loss, petechial hemorrhages on the third eyelid, anemia, and edema under abdomen, scrotum and limb area. Few animals in later stages show nervous sign and death (Ngaira et al., 2002). Working donkeys are the neglected animals and no research was conducted on molecular identification of Trypanosoma species of donkeys in Pakistan. Comparative effect of trypanosomiasis on hematology and serum values of infected as well as healthy donkeys has not been also observed. Therefore, in continuation with the existing knowledge, the present study was planned to jot down:

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a. Microscopic prevalence of trypanosomiasis in donkeys
b. Hematological examination of trypanosoma infected and non-infected animals
c. Serological analysis of trypanosome infected and non-infected animals
d. Molecular identification of different Trypanosoma species

Materials and Methods

Study Area

Gujranwala, Gujrat and Mandi Bahaudin are three districts of Punjab, Pakistan. Gujranwala is located between 32.16 latitude and 74.19 longitude while Gujrat is located between 32.57 latitude and 74.08 longitude; whereas, Mandi Bahaudin is located 32.58 latitude and 73.48 longitude.

Sample Collection

The study population consisted of working donkeys of all age groups that were selected randomly from three districts of Punjab including Gujranwala, Gujrat and Mandi Bahaudin. Blood samples (8 mL) from 657 donkeys were collected from jugular vein aseptically through sterile syringe following the guideline of international animal ethics and welfare committee. About 4 mL blood was transferred into tubes containing EDTA (ethylene diamine tetraacetate) for hematological study while the rest was put in EDTA-free clot activator tubes for serum separation.

Microscopic Examination

A drop of blood was placed on a clean glass slide and a coverslip placed on it, allowing the blood to spread as a thin layer of cells. This was then examined under microscope to observe motile trypanosomes. For morphological examination, thin smear then drawn out with another slide and air dried. Fixation of the slide was carried out with methanol for 2-3 min and 5% Giemsa stain was used for staining purpose followed by rinsing with distilled water (pH 7.2). The slide was then air dried and examined under a high power 100X microscope by using immersion oil.

Hematological and Serum Biochemical Examination

Sysmex hematologic analyzer was used to examine the hematological profile of trypanosome infected and non-infected healthy animals. Healthy animals were free from blood parasites with body condition score ≥2. Screening panel included total erythrocyte count (TEC), total leucocyte count (TLC), differential leucocyte count (DLC), packed cell volume (PCV), hemoglobin concentration (Hb), erythrocyte sedimentation rate (ESR), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

The serum biochemical profile was analyzed for total proteins (TP), albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) by commercially available kits in the RX Monza of Randox RX Series.

PCR Amplification

Multiplex PCR assay was performed for molecular detection of Trypanosoma species in donkeys. DNA was extracted from the blood following Ghatak et al. (2013) and amplification was carried out in thermal cycler (2720 Applied Biosystem®). Samples were loaded in thermal cycler containing Trypanosoma species specific primers master mix solution and DNA templates then the conditions of thermal cycler were adjusted according to optimization levels.

Primers

The species-specific primer sequences used are shown in Table 1.

PCR amplification solution: Total 80 µL amplification solution was prepared which contained 25 µL water, 45 µL master mix, 4 µL extracted DNA and 1 µL of each primer.

Thermal Cycler Conditions

The PCR tubes having PCR mixture were placed into thermocycler. Initial denaturation was done at 94°C, followed by 39 cycles of denaturation, annealing, and extension, a final extension was carried out at 72°C and the amplified sample was stored at 4°C. The duration and different levels of temperature used are given in Table 2. The extension was carried out at 72°C for 10 min. PCR products were held at 4°C until separated by electrophoresis on a 1% agarose gel and visualized under a UV Trans-illuminator.

Statistical Analysis

Data were analyzed using a randomized design. F-test was used to compare data from positive and negative samples at 5% level of significance (α=0.05). The results were considered significant if F < 0.05 level. Chi-square test was used to see if there exists any significant difference between different treatment groups.

Results

Prevalence of Trypanosomes

Prevalence of trypanosomiasis was 8.83% in donkeys (n=657), detected positive under a microscope. Gujranwala had the highest prevalence 11.58% of trypanosomiasis (Table 3); whereas, Mandi Bahaudin had the least prevalence 5.39% but they were non-significant.
Table 1: Species-specific primer sequence

<table>
<thead>
<tr>
<th>Trypanosoma vivax (Cox, 2007)</th>
<th>TVW1: 5’-CTGATGTCTCATTGTCAGCCAC-3’</th>
<th>TVW2: 5’-CCACCAAGAACAAACCTGTA-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypanosoma congolense (Almeida et al., 1998)</td>
<td>GOL5’-GAGAAGCGCGCATTITGCGATTTC-3’</td>
<td>GOL5’-GACAAAACAAATCCGCACACCAT-3’</td>
</tr>
<tr>
<td>Trypanosoma evansi (Birhanu et al., 2015)</td>
<td>RoTat-F: 5’-GCGGGGTGTTAAGACATAA-3’</td>
<td>RoTat-R5’-ATTATGTCGCGTGTGCG-3’</td>
</tr>
</tbody>
</table>

Table 2: Thermal cycler conditions

<table>
<thead>
<tr>
<th>Solution/Primer</th>
<th>Process</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOL Primer 314 bp</td>
<td>Initial denaturation</td>
<td>94°C</td>
<td>4 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>50°C</td>
<td>40 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>TVW Primer 150 bp</td>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>40 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>RoTat Primer 205 bp</td>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>59°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Infinite hold</td>
<td>4°C</td>
<td>30 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Prevalence of trypanosomiasis in three districts

<table>
<thead>
<tr>
<th>District</th>
<th>Animals screened</th>
<th>Infected</th>
<th>Prevalence (%)</th>
<th>Chi-square value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gujranwala</td>
<td>259</td>
<td>30</td>
<td>11.5%</td>
<td>5.000</td>
<td>0.172</td>
</tr>
<tr>
<td>Gujrat</td>
<td>231</td>
<td>19</td>
<td>8.23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandi Bahauddin</td>
<td>167</td>
<td>9</td>
<td>5.39%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>657</td>
<td>58</td>
<td>8.83%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant if P<0.05

Prevalence of Trypanosomiasis among Sex and Age Groups

Trypanosomiasis was more prevalent in males 9.8% than females 7.88% (Table 4). In this study, males and females under one year of age had the least prevalence (male 6.32% and female 6.67%); whereas, the highest prevalence was observed in the animals with age more than 6 years (male 12.33% and female 9.76%). Pregnant dry 13.3%, pregnant 16.6% is not significantly higher than non-pregnant dry and lactating.

Morphology of RBC

In this study, trypanosomes were observed in Giemsa stained slides under the microscope. Red blood cells of different shapes were seen which included macrocyte (Fig. 1), microcyte (Fig. 2), acanthocyte (Fig. 3), dacrocyte (Fig. 4) and bizarre shaped cells (Fig. 5).

Serum and Hematological Values of Infected and Healthy Animals

Values of various hematological and serum parameters were compared between infected and non-infected animals.

Table 4: Distribution of experimental animals according to sex and age (Chi Square analysis)

<table>
<thead>
<tr>
<th>Animals screened</th>
<th>Infected</th>
<th>Prevalence (%)</th>
<th>Chi-square value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>327</td>
<td>32</td>
<td>9.8%</td>
<td>0.742</td>
</tr>
<tr>
<td>Female</td>
<td>330</td>
<td>26</td>
<td>7.88</td>
<td></td>
</tr>
</tbody>
</table>

Significant if P<0.05

Table 5: Mean ±SEM values of hematological and serum biochemical parameters of infected and healthy donkeys

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Infected donkeys</th>
<th>Healthy donkeys</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin g/dL</td>
<td>6.34±2.34*</td>
<td>10.7±0.933</td>
<td>0.0001</td>
</tr>
<tr>
<td>RBC million/cm³</td>
<td>4.20±0.57*</td>
<td>6.28±0.49</td>
<td>0.0001</td>
</tr>
<tr>
<td>Packed cell volume %</td>
<td>19.26±2.94*</td>
<td>33.70±2.33</td>
<td>0.0001</td>
</tr>
<tr>
<td>MCV μm³</td>
<td>45.26±7.83*</td>
<td>54.12±4.89</td>
<td>0.0001</td>
</tr>
<tr>
<td>MCH pg</td>
<td>14.87±2.95*</td>
<td>16.59±2.96</td>
<td>0.0028</td>
</tr>
<tr>
<td>MCHC g/dL</td>
<td>34.1±8.3</td>
<td>31.98±30</td>
<td>0.0973</td>
</tr>
<tr>
<td>Total leucocyte 10³/cm³</td>
<td>15.26±3.35*</td>
<td>11.63±1.50</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lymphocyte %</td>
<td>63.45±4.37*</td>
<td>40.9±2.87</td>
<td>0.0001</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>28.8±3.81*</td>
<td>51.3±3.02</td>
<td>0.0001</td>
</tr>
<tr>
<td>Monocyte %</td>
<td>4.42±1.72</td>
<td>4.57±1.15</td>
<td>0.2139</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>4.00±1.24*</td>
<td>3.10±0.79</td>
<td>0.0014</td>
</tr>
<tr>
<td>Alkaline phosphatase U/L</td>
<td>396.82±8.26,83*</td>
<td>337.22±44.51</td>
<td>0.0004</td>
</tr>
<tr>
<td>Total protein g/dL</td>
<td>7.88±1.33*</td>
<td>6.77±1.24</td>
<td>0.0058</td>
</tr>
<tr>
<td>Albumin g/dL</td>
<td>2.71±0.47*</td>
<td>3.22±0.77</td>
<td>0.0045</td>
</tr>
<tr>
<td>Aspartate aminotransferase U/L</td>
<td>387.00±42.72*</td>
<td>242.8 ±42.18</td>
<td>0.0001</td>
</tr>
<tr>
<td>Alanine aminotransferase U/L</td>
<td>24.82±6.00*</td>
<td>19.01±7.06</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

Significant if P<0.05

Hemoglobin, red blood cells count, packed cell volume (PCV) and neutrophil count were significantly decreased in infected animals as compared to healthy animals (Table 5); whereas, total leucocyte count and lymphocyte count increased significantly. All the parameters including total proteins alkaline phosphatase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly higher in the infected animals as compared to healthy animals while, albumin was significantly decreased in infected animals as compared to in healthy animals.

Prevalence of Trypanosomiasis through PCR

Trypanosoma evansi, T. congolense and T. vivax can be transferred mechanically or biologically (Abenga et al., 2002) so there might be the presence of different trypanosome species in the naturally infected animals.

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To identify the prevalence of each *Trypanosoma* species in trypanosomiasis infected animals, three species-specific primers were used for *T. vivax*, *T. congolense* and *T. evansi*. *Trypanosoma evansi* was targeted (Fig. 6) by RoTat at 205 base pair; whereas, *T. vivax* and *T. congolense* were not observed in these infected donkeys (Table 6). Our study showed that *T. evansi* was only and the most prevalent specie in the working donkeys of Pakistan. It also indicated that only *T. evansi* was mechanically transmitted in Pakistan; whereas, *T. congolense* and *T. vivax* were neither transmitted mechanically nor biologically.

**Discussion**

This study was conducted to investigate the molecular identification and prevalence of trypanosomiasis and its effects on hematological and biochemical parameters in donkeys. Blood samples were collected from 657 donkeys of three districts: Gujranwala, Gujrat, and Mandi Bahaudin in Punjab province. Blood screening indicated 8.83% trypanosomiasis infection in donkeys; whereas, Multiplex PCR showed that only *T. evansi* is prevalent in Pakistan. This study will help in developing strategies to curb the trypanosomiasis.

Prevalence of trypanosomiasis was observed to be 8.83% in above mentioned three districts. No district wise data was available to compare these findings. Previously, Abbasi *et al.*, 2014 reported 8.46% trypanosomiasis in donkeys. Hussain *et al.*, 2016 reported 6.71% trypanosomiasis in donkeys. However, Hassan *et al.* (2006) reported 3.3% trypanosomiasis prevalence among equines in Punjab. This variation indicates the possibility of low trypanosomiasis pressure in other districts of Punjab than the ones in the current study.

### Table 6: Prevalence of trypanosomiasis using PC

<table>
<thead>
<tr>
<th></th>
<th>Trypanosoma evansi</th>
<th>Trypanosoma congolense</th>
<th>Trypanosoma vivax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected samples</td>
<td>58</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prevalence</td>
<td>100%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Fig. 1:* Microcyte

*Fig. 2:* Macrocytic

*Fig. 3:* Acanthocyte

*Fig. 4:* Dacrocyte (Tear droplet cells),

*Fig. 5:* Bizarre shape (Giemsa Stained- 1000X)

*Fig. 6:* PCR product from amplification of DNA taken from the blood of donkeys. The primers used are specific for *Trypanosoma evansi*, *Trypanosoma vivax*, and *Trypanosoma congolense*. Band at 205bp (base pair) indicated *Trypanosoma evansi*. L is the molecular marker, +ve and –ve are positive and negative control; 19, 82, 83, 84, 92, 17, 16, 21, 22, 23 are amplified DNA of infected samples.

*T. evansi* was targeted (Fig. 6) by RoTat at 205 base pair; whereas, *T. vivax* and *T. congolense* were not observed in these infected donkeys (Table 6). Our study showed that *T. evansi* was only and the most prevalent specie in the working donkeys of Pakistan. It also indicated that only *T. evansi* was mechanically transmitted in Pakistan; whereas, *T. congolense* and *T. vivax* were neither transmitted mechanically nor biologically.
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There was no significant difference in the prevalence of trypanosomiasis with sex, age and reproductive status of animals. However, a non-significant higher prevalence of trypanosomiasis was observed in male and adult working donkeys than females and young ones. Reason for higher incidence of disease in male animals than females could be due to the fact that male animals travel from one place to another to provide transportation services more than females. Thus, males have a higher probability of acquiring an infection. Frequent travelling can also compromise their immune response to infection due to the stress of fatigue (Kassa et al., 2011). Hussain et al. (2016) reported no significant difference in trypanosomiasis among the sex and age groups of donkeys in Pakistan. Our result showed agreement with the Bogale et al. (2012) who also observed a non-significant higher prevalence in male animal than females. Mekibib et al., 2010 observed that sex and age had no influence on prevalence of trypanosomiasis.

Red blood cells of different shapes such as macrocyte, microcyte, acanthocyte, dacrocyte and bizarre shaped cells were seen in infected donkeys. No literature was available to compare effects of trypanosomes on red blood cell’s morphology for three districts of Punjab. However, Silva et al. (1995) reported the morphological alteration of red blood cells by Trypanosoma species in dogs and horse. Acanthocyte, microcyte, macrocyte, dacrocyte, microspherocyte, anisocytosis and bizarre shaped red blood cells appeared in the dogs and horse infected with natural trypanosomiasis. Morphological alteration in the red blood cells might be due to liver abnormalities since; this organ plays a major role in removing blood parasites (Abdulmajeed et al., 2007). Hussain et al., 2016 has reported macrocyte, microcyte, elliptical cells and tear droplet cell in camel infected with natural trypanosomiasis.

Our study showed significant alteration in hematological and serum parameters in the trypanosomiasis infected donkeys. Cadioli et al. (2006) in donkeys, Hilali et al. (2006) in buffalo calves, Chamoud et al. (2010) in mice, Ohaeri and Eluwa (2011) in sheep and Padmaja (2012) in camels reported variation in the hematological parameters due to trypanosomiasis in infected animals. In trypanosomiasis, the main pathological changes in blood are the reduction in hemoglobin and hematocrit values. Lashing movement of Flagella, hemodilution (Reddy et al., 2016), hemolysis of RBCs (Rossi et al., 2017), erythro- phagocytosis (Ohaeri and Eluwa, 2011) and metabolites released from trypanosomes (Naessens, 2006) are the main causes that change hematological parameters in infected animals. Sialidase enzyme released from trypanosomes breaks sialic acid on cell membrane of erythrocytes and make the erythrocyte more susceptible for phagocytosis that in turn produce anemia in the infected animals (Ohaeri and Eluwa, 2011). Padmaja (2012), Hussain et al. (2016) reported a significant reduction in RBCs, hemoglobin, and hematocrit in trypanosomiasis infected animals which were in complete agreement with our findings.

Leucocytosis with lymphocytosis was found in positive animals in our study which showed complete agreement with Sivajothi et al. (2015) who reported significant increase in total leucocyte count in the infected rabbit. Ohaeri and Eluwa (2011) reported an increase in leucocyte in infected sheep, goat, and cattle wherein lymphocytosis was observed in all these cases. In contrast to all these, Padmaja (2012), Chaudhary and Iqbal (2000) reported decreased lymphocyte count in infected camels. Reason behind this decreased lymphocyte count could be the immune suppression. Secondly, these reports could have been about initial stages of infection where neutrophil increased and lymphocyte decreased.

Our study reported a significant higher value of serum total proteins in the infected animals which might be due to released immunoglobulin from the defense system in response to disease (Taylor and Authić, 2004; Baral et al., 2007). Increase in serum protein could also be due to the release of tissue specific enzyme and disruption of the cell membrane in the infected animals. Several studies reported significant increase in total protein in the trypanosomiasis infected animals e.g., Orhue et al. (2005) reported this in rabbit, Gutierrez et al. (2005) in camel and Hilali et al. (2006) in buffalo calves, Hussain et al. (2016) in donkeys and Oparah et al. (2017) in Nigerian donkeys. Decreased albumin in our study was also reported by Orhue et al. (2005), Ohaeri and Eluwa (2011). The reasons of hypoalbuminemia were hepatic injuries due to centrilobular degeneration and hypoxia in trypanosomiasis (Hussain et al., 2016). Decreased albumin level maintains osmolarity that causes compensation in hyerglobulinemia (Ahmad-Hamedani et al., 2014). Edema in the dependent part of the body in trypanosomiasis might be due to decreased albumin level (Enwezor and Sackey, 2005).

In our study, all the serum enzyme including alkaline phosphatase ALP, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly higher in the infected animals. Takeet and Fagbemi (2009), El-Baky and Salem (2011) showed complete agreement with our study who reported a significant rise in alkaline phosphatase in infected animals. Oparah et al. (2017) reported significant rise in ALT, AST and ALP in Nigerian donkeys infected with trypanosomiasis hepatic necrosis in naturally infected camel with trypanosomiasis might be the reason of increased ALP, AST and ALT (El-Baky and Salem, 2011). A significant increase in ALP, AST, and ALT was also reported in infected rabbit by Takeet and Fagbemi, (2009). Taiwo et al. (2003), Sivajothi et al. (2015), Hussain et al. (2016) also reported significant rise AST and ALT in infected animals. Tissue damage (necrosis) and inflammatory changes in liver, kidney, heart, and muscle cause a significant increase in these enzymes. Host immune system lysed trypanosomes in different stages of infection which might also cause an increase in ALT and AST (Taiwo et al., 2003; Takeet and Fagbemi, 2009).
To the best of our knowledge, this is the first ever report on prevalence of trypanosomiasis in donkeys in Pakistan using species-specific primers. Since, *Trypanosoma evansi* was the only species detected by PCR, therefore, more emphasis should be laid on *T. evansi* infection, control and treatment in future in Pakistan. Hematological parameters were altered significantly in infected animals that ultimately affected the donkey health and productivity of animals. Precautionary measures should be taken to prevent flies (vectors) from biting the animals. Epidemiologists and policy makers are suggested to devise species and area specific mitigation measures against trypanosomiasis infection in working equines. PCR based (species specific) investigation of trypanosomiasis in other equine and livestock species are also strongly recommended.

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