Molecular detection and therapeutic study of *Trypanosoma brucei evansi* from naturally infected horses in Punjab, Pakistan

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Abstract

Trypanosomiasis is one of the severe pathogenic infections, caused by several Trypanosoma species, affecting both animals and humans, causing substantial economic losses and severe illness. The objective of this study was to determine the molecular diagnosis and the risk factors associated with trypanosomiasis in District Jhang, Punjab, Pakistan. For this purpose, blood samples were randomly collected from 200 horses. A predesigned questionnaire was used to collect data on risk factors before the sample collection. The microscopy examination through Giemsa staining, formol gel test and PCR techniques were used to find the prevalence. The prevalence was recorded as 22.5% with microscopy examination, 21% through formol gel test and 15.5% with PCR based results. Analysis of risk factors associated with *Trypanosoma brucei evansi* occurrence was carried out using Chi-square test. It showed the prevalence of *Trypanosoma brucei evansi* was significantly (p<0.05) associated with sex, age, rearing purpose and body condition whereas non-significantly (p>0.05) with insects control practices. This study supports the idea that PCR is a sensitive, robust and more reliable technique to diagnose trypanosomiasis. It was concluded that *Trypanosoma brucei evansi* is widely prevalent in Jhang (Pakistan), highlighting a dire need to develop control strategies and education programmes to control this disease in developing countries.

Key words: equines, prevalence, *Trypanosome brucei evansi*, therapeutic trial, horses
Introduction

Horses are grown in Pakistan for numerous purposes, including racing, farming, transportation, companionship, exhibition and breeding (Tehseen et al. 2017). According to economic survey of Pakistan in 2017, there were 4.9 million working equines in Pakistan. Trypanosomiasis (Surra) caused by hemoprotozoan parasite Trypanosoma brucei evansi, is one of the leading veterinary concerns in the world due to its drastic effects on the health, performance and production of the affected animals. The ailment has detrimental effects on animal health, resulting in immense economic losses (Kandeel and Al-Taher 2021).

Trypanosoma brucei evansi is a hemoflagellate that is spread mechanically by several species of blood sucking flies (mainly Tabanids and Stomoxys), but in Latin America, the vampire bat (Desmodus rotundus) is a vector and reservoir host of Trypanosoma brucei equinum causing disease, ‘Mal de Caderas’ (Stephen 1986). The dogs and cats after getting infected with biting flies serve as the source of transmission for the Trypanosomes in the animal settings with a potential risk to humans (Cardinal et al. 2008). The protozoon is also found in the blood, body fluids and in the tissues of certain organs (Kennedy 2013). Trypanosoma brucei evansi was first to be described by Griffith Evans in the blood samples of equines and dromedaries camels in 1880 (Aregawi et al. 2019). The word “surra” comes from the Indian language and indicates “rotten” which describe the condition of the animals after chronic disease development (Desquesnes et al. 2013).

Trypanosoma brucei evansi infects a wide variety of animal hosts, but equids, camels and bovines are most adversely affected animals. It has been diagnosed and depicted in Africa, Asia, South America, and the Middle East (Berlin et al. 2012). The disease was recorded in different regions of Pakistan (Aslam et al. 2010). In livestock, several species of trypanosomes are present, namely T. brucei, T. vivax, T. evansi, T. congolense (Baticados et al. 2012).

Clinically, the disease is characterized by emaciation, reduced fertility, neurological signs and immunosuppression combined with anemia and ultimately death in both domestic and wild vertebrates (Tehseen et al. 2015). The symptoms usually appear in horses after 15-20 days of the incubation period associated with fever, emaciation, anemia, fatigue and laziness depending on disease intensity (Sobia et al. 2018). Nervous signs may also develop during the terminal phase of the infection in horses infected with Trypanosoma brucei evansi (Eregat et al. 2020). Horses and camels are highly prone to trypanosomiasis and may die within a few weeks or in a month (Yadav et al. 2014).

Blood smear microscopy is the easiest and most frequently used screening test to diagnose trypanosomiasis for which a thin blood smear slide prepared is stained with Giemsa stain and observed under a light microscope at 100X magnification. Trypanosoma brucei evansi is monomorphic, slender-shaped, with an elongating membrane and well-developed free flagellum present outside the cell surface (Muieed et al. 2010). Morphologically, Trypanosoma brucei evansi is a hemoparasite measuring about 14-29 μm in length and 1.5-3.45 μm in width and contains nucleus (Hassan-Kadle et al. 2019). However, the rate of parasitemia is usually lower and fluctuates notably in the chronic phase, so the involvement of trypanosome can go unreported. Therefore, a more robust and more precise technique needs to be used. In recent years, DNA-based technologies including PCR have been used for the detection of trypanosomiasis (Simwango et al. 2017). Other serological diagnostic tests such as card agglutination test and enzyme-linked immunosorbent assay are also used for detection of Trypanosoma brucei evansi infection (Jiang et al. 2018).

The PCR is a very tactful and precise approach for diagnosing surra and has been frequently used in certain countries (Baticados et al. 2012). In addition, the actual sensitivity and specificity of such techniques depend directly on the blood volume examined and the examiner’s skills and experience (Elshafie et al. 2013). This method has also been used for large-scale analysis of trypanosomes samples (Simwango et al. 2017). Keeping in view the severity of the disease and economic losses caused by it, the present study was designed to find its prevalence, associated risk factors and therapeutic trial of different drugs.

Materials and Methods

The Ethics Committee of the CVAS, Jhang (Sub Campus University of Veterinary & Animal Sciences, Lahore), initially approved the present thesis (No. CVAS/11516 dated 07-01-2020) and then the final approval of the material was accorded by the Directorate of Advanced Studies (DAS/537 dated 15-06-2020) of the University. Verbal and written consents were obtained from each owner/attendant before collecting blood samples and delivering the treatments to their animals.

A total of 200 blood samples were randomly collected from different breeds of horses from district Jhang Punjab, Pakistan. About 5 ml of the blood sample was collected from the jugular vein of the animals by using 10 ml disposable syringe following aseptic protocol and immediately transferred to properly labelled purple-capped vacutainer (BD Vacutainer® spray-
Table 1. The overall prevalence of *Trypanosoma brucei evansi* in horses based on different tests in District Jhang, Pakistan.

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of horses tested</th>
<th>Positive</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>200</td>
<td>45</td>
<td>22.5%</td>
</tr>
<tr>
<td>Formol gel test</td>
<td>200</td>
<td>42</td>
<td>21.0%</td>
</tr>
<tr>
<td>PCR based prevalence</td>
<td>200</td>
<td>31</td>
<td>15.5%</td>
</tr>
</tbody>
</table>

A total 18 positive horses were randomly divided into three groups A, B, and C for therapy to evaluate the comparative efficacy of drugs. The horses of group A were treated with Tryban® (Quinapyramine sulphate) @ 5mg/kg body weight intramuscularly (UM Enterprises). The horses of group B were treated with Diminazene® (Diminazene diacetate) @ 3.5-7mg/kg intramuscularly for 3 days (Star laboratories Pvt. Ltd) whereas Neem leaves powder (*Azadirachta indica*) @ 60 g daily PO was used to treat group C against trypanosomiasis. After elapsing of 7 days post-treatment, blood samples were taken again from the treated animals to recheck the status of animal.

The following formula was employed to calculate the efficacy of used drugs.

Therapeutic efficacy % = No. of recovered animals / No. of total animals × 100

The data thus obtained were analyzed using Pearson Chi-square test for the parameters pertaining to the prevalence of the protozoan while the therapeutic efficacy of the selected drugs was determined by using the SPSS software version 21.

**Results**

Out of 200 blood samples of horses screened for *Trypanosoma brucei evansi* infection, 45 (22.5%) were detected positive through Giemsa stain, 42 (21.0%) through formol gel test and 31 (15.5%) based on PCR as shown in Table 1. The microscopy was not precisely able to distinct *Trypanosoma evansi* spp. in the blood sample. It may be due to visual confusion in the identification of trypanosome species. Positive samples detected through PCR on electrophoresis gel are shown below in Fig. 1.

Analysis of risk factors associated with *Trypanosoma brucei evansi* occurrence was carried out using Chi-square statistics (Table 2). It showed the prevalence of *Trypanosoma brucei evansi* was significantly associated with the various risk factors. The prevalence was significantly higher (p=0.011) in males as compared with the females. The prevalence of the disease was higher in animals above 4 years of age than in the younger animals. There was a significant difference in prevalence among age groups (p=0.027). The prevalence of disease was significantly (p=0.032) higher...
recovered successfully. The efficacy of Tryban® (Quinapyramine sulphate) was 100%. The group B treated with Diminazine aceturate along with supportive treatment, four animals recovered. The efficacy of Diminazine aceturate was 66.6%. In group C treated with Neem leaves powder (Azadirachta indica) @ 60 g daily PO along with same supportive therapy, three animals recovered without any side effects. The efficacy of Neem leaves powder (Azadirachta indica) was 50% (Table 3). The drug Quinapyramine sulphate and Neem leaves powder (Azadirachta indica) was very effective in removing the Trypanosomes from blood with less side effects.

Table 2. PCR based prevalence of Trypanosoma brucei evansi and its associated risk factors in horses of Jhang district, Pakistan.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Level</th>
<th>No. of horses tested</th>
<th>Positive</th>
<th>Prevalence %</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>120</td>
<td>23</td>
<td>19.16</td>
<td>0.011*</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>80</td>
<td>8</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&gt;4 years</td>
<td>100</td>
<td>19</td>
<td>19.0</td>
<td>0.032*</td>
</tr>
<tr>
<td></td>
<td>1-4 years</td>
<td>70</td>
<td>9</td>
<td>12.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 1 year</td>
<td>30</td>
<td>3</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Purpose</td>
<td>Transportation</td>
<td>140</td>
<td>27</td>
<td>19.28</td>
<td>0.027*</td>
</tr>
<tr>
<td></td>
<td>Breeding</td>
<td>60</td>
<td>4</td>
<td>6.66</td>
<td></td>
</tr>
<tr>
<td>Body Condition</td>
<td>Good</td>
<td>107</td>
<td>5</td>
<td>4.67</td>
<td>0.012*</td>
</tr>
<tr>
<td></td>
<td>Emaciated</td>
<td>93</td>
<td>26</td>
<td>27.95</td>
<td></td>
</tr>
<tr>
<td>Insects control</td>
<td>No</td>
<td>143</td>
<td>24</td>
<td>16.78</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>57</td>
<td>7</td>
<td>18.28</td>
<td></td>
</tr>
</tbody>
</table>

* Values indicate statistically significant difference (p<0.05) associated with various risk factors

in working animals as compared with the breeding animals. The disease was highly associated with emaciation (p=0.012) as compared with good body condition. However, the insects control practices did not represent a significant role (p=0.25).

Therapeutic efficacy of all compounds was assessed based on disappearance or amelioration of clinical signs, absence of Trypanosoma brucei evansi parasite in blood examination and PCR on day 7 post-treatment. The results of therapeutic trial were depicted in Table 2. In group A, all the six animals were treated with Tryban® (Quinapyramine sulphate) @ 5mg/kg on alternative days along with supportive therapies and recovered successfully. The efficacy of Tryban® (Quinapyramine sulphate) was 100%. The group B treated with Diminazine aceturate along with supportive treatment, four animals recovered. The efficacy of Diminazine aceturate was 66.6%. In group C treated with Neem leaves powder (Azadirachta indica) @ 60 g daily PO along with same supportive therapy, three animals recovered without any side effects. The efficacy of Neem leaves powder (Azadirachta indica) was 50% (Table 3). The drug Quinapyramine sulphate and Neem leaves powder (Azadirachta indica) was very effective in removing the Trypanosomes from blood with less side effects.
Table 3. Drug trials and their efficacy percentage.

<table>
<thead>
<tr>
<th>Group</th>
<th>Therapeutic agents, their composition, Dosage and route of administration</th>
<th>No. of horses treated</th>
<th>No. of horses recovered</th>
<th>Drug efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tryban® (Quinapyramine methyl sulphate) @ 5mg/kg body weight SC (UM Enterprises)</td>
<td>6</td>
<td>6</td>
<td>100%</td>
</tr>
<tr>
<td>B</td>
<td>Diminazene® (diminazene diaceturate) @ 3.5-7mg/kg IM for 3 days</td>
<td>6</td>
<td>4</td>
<td>66.6%</td>
</tr>
<tr>
<td>C</td>
<td>Neem leaves powder (Azadirachta Indica) @ 60g daily PO for 7 days</td>
<td>6</td>
<td>3</td>
<td>50%</td>
</tr>
</tbody>
</table>

Discussion

In the present study the prevalence of trypanosomiasis by microscopy in horses was 22.5%. Several studies in Pakistan reported the prevalence of Trypanosoma brucei evansi in different species. The prevalence of trypanosomiasis was 13.7% in Sindh, Pakistan (Shah et al. 2004). The difference in rate of infection of trypanosomiasis in Sindh, Pakistan may be due to difference in environmental conditions and climate changes as compared with Punjab. Similarly, 2% prevalence was reported in horses and donkeys by thin smear blood microscopy from Gujranwala, Pakistan (Aslam et al. 2010).

The prevalence of Trypanosoma spp. has been reported by several countries. The 56.25% prevalence of Trypanosoma brucei evansi in camels was reported in Iraq (Al-Amery et al. 2017) and 17.61% from Iran (Mirshekar et al. 2017) and 0.96% from Egypt (Henidy et al. 2019), 15.25% prevalence in buffaloes from India (Gangwar et al. 2019), 9.1% in horses and donkeys from Gambia (Pinchbeck et al. 2008) and 34.4 percent in camel samples 6.8 percent of donkey and 7.7% of dog samples were found positive by thin blood smear microscopy from India (Mandovera et al. 2008). Our study findings are consistent with these studies that Trypanosoma brucei evansi is widely prevalent in these countries, like in Pakistan.

Formol-gel test is a non-specific biochemical test that has been used to detect the chronic infection. However, researchers have attempted to report the prevalence of Trypanosoma spp. through this test. Sobia et al. (2018) revealed 51.1% prevalence Trypanosoma species through formol gel test from cattle, goat, sheep, donkeys and camels from Pakistan. Similarly, 24% equines were found to be positive through the formol gel test from Lahore, Pakistan (Tehseen et al. 2015, Aslam et al. 2010). In the present study, we found 21% prevalence of trypanosoma from district Jhang.

In the current study, it has been found that PCR-based techniques are more sensitive and precise for the identification than microscopy and other techniques. In addition, this test was also used in the large scale for analysis of trypanosomes samples (Desquesnes et al. 2013). The results of the current research were also accordance to the findings of Alanazi et al. (2018), who found that PCR was more specific for the detection of trypanosomal antigens. In another study (Henidy et al. 2019), it was shown that the use of PCR in the diagnosis of trypanosomiasis is more emphatic and precise, particularly in cases with low infection rates, as well as potentially valuable in epidemiological studies. Similarly, Aslam et al. (2010) also found that PCR test sensitivity for the detection of trypanosomes in horses and donkeys blood samples was higher than Formol Gel test. In the present study, PCR based prevalence of Trypanosoma brucei evansi is 15.50%. Some of the samples that were positive in microscopy and formol gel test, were negative when analyzed with PCR. These findings are consistent with (Baticados et al. 2012) who also found that PCR is more precise and sensitive technique than other tests.

In the present study the prevalence of trypanosomiasis is higher in older animals than the younger animals. Our research is in accordance with the study of Elhaig et al. (2018) who also indicated the greater prevalence in old horses as compared with adult animal. This may be attributed to extreme stress by using them to transport goods from one location to another or due to poor management. In contrast to our study Tehseen et al. (2017) found the non-significant disparity between age groups, indicating that all age groups are equally affected by trypanosomiasis (surra). In the present study, sex-wise prevalence is higher in male horses than female horses that is in accordance with the findings of Nurcahyo et al. (2019) who also found a higher prevalence in males as compared with female. Our findings are inconsistent with Hasan et al. (2006) who found both sex are equally affected. The lower prevalence in breeding horses at farm as compared to transport horses may be related to effective management, good nutrition and accessibility of veterinarians. Horses with poor body condition and used for transportation purpose at brick-kilns showed higher prevalence for Trypanosoma brucei evansi that may be due to
stress, work overload, poor nutrition, and increased burden of other infections. Our results are consistent with findings of Hasan et al. (2006), who found a higher prevalence in horses that were emaciated and used for draft purposes.

After confirmation of infection through PCR, all animals were treated with Diaminazine aceturate, Quinapyramine sulphate, and Neem leaves powder (Azadirachta indica). Quinapyramine sulphate had an outstanding response as treatment. Four out of 6 animals were cured after the first dose and two animals were cured after the second dose. The efficacy of the drug was considered to be 100%. Our results are inconsistent with the study of Hota et al. (2019) who used Quinapyramine sulphate in India, for the treatment of T. evansi and Matovu et al. (2020) who used it for the treatment of “surra” in horses and camels and obtained highest efficacy. Diminazene diaceturate was also found to have a good effect on the disease and showed to have 66.6% efficacy, but the drug showed adverse reaction as reported earlier (Raffery et al. 2018). Neem leaves powder (Azadirachta indica) was found to be 50% effective in curing of animals with less toxic effects. These results are similar with the study reported by Omoja et al. (2011). The findings of Mahboob et al. (2008) are also consisted with our study. They used Azadirachta indica dried leaves at the same dose in horses infected with strongylosis and cured the animal. The efficacy of 10% neem water extract against nematodes in sheep was observed by Amin et al. (2011) and significant declines in EPG count were observed. The anti-trypanosomal activity of Azadirachta indica may be due to the presence of secondary metabolites in the plant capable of producing radicals that act against the metabolism of the parasite (Enyanwu et al. 2011). The pathological effect of trypanosoma is mediated by release of cytokines and nitric oxides. Active Neem (Azadirachta indica) compounds such as phenol act to neutralise poisonous chemicals, thereby break the life cycle of the organism. Ajabe et al. (2018) also suggested that the failure of the extract to completely kill the parasite may be attributed to the low levels of active compounds in the aqueous extract.

Conclusion

Trypanosomiasis is highly prevalent in Jhang in subacute and chronic form. The PCR test is more sensitive technique for identification trypanosomal infection in horses compared to Formol Gel test and Thin Blood Smear microscopy. Quinapyramine sulphate can be used effectively to treat the infected animals. Neem leaves powder has proved to possess anti trypanosomal potentials and can be used in case of unavailability of a drug, especially in ruler areas as a natural remedy. This study suggested the higher rural authorities and Government officials to make a policy for the control of this disease.

Acknowledgements

Lab consumables were purchased from a research Project No. PARB 660, 2018 being executed in the discipline of Parasitology of College of Veterinary and Animal Sciences, Jhang-Pakistan.

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