Efficacy of Some Trypanocidal Drug Against *Trypanosoma equiperdum* OVI in Experimentally Infected Mice in Debre Zeit, Ethiopia

1Beletu Habte, 1Abrha Bsrat, 2Hagos Ashenafi and 1Fikru Regassa

1College of Veterinary Medicine, Mekelle University, P.O. Box 231, Mekelle, Ethiopia
2Addis Ababa University College of Veterinary Medicine, P.O. Box 1176, Addis Ababa, Ethiopia

Abstract: Trypanocidal drugs remain the principal method of animal Trypanosomiasis control in most African counties. However, there is growing concern that future effectiveness may be severely curtailed by widespread drug resistance. This study was therefore, conducted to assess the efficacy of diminazene diaceturate and cymelarsan against *Trypanosoma equiperdum* OVI South Africa strain in experimentally infected mice. Sensitivity study was conducted using range doses of diminazene diaceturate (3.5mg/kg, 7.0mg/kg, 14mg/kg and 28mg/kg) and cymelarsan (0.25 mg/kg, 0.5 mg/kg 1.0 mg/kg and 2.0mg/kg) using 50 mice each being grouped into 10 having 5 mice each. Diminazene diaceturate at doses of 3.5mg/kg, 7.0mg/kg, 14mg/kg failed completely to cure but only 1(20%) mice out of 5 was survive up to 60 days treated with diminazene diaceturate at dose of 28mg/kg body weight. Cymelarsan at dose of 0.25mg/kg body weight failed completely to cure mice but a dose of 0.50mg/kg body weight, only 2(40%) mice were cure. All mice treated at higher dose of the cymelarsan 1.0mg/kg and 2.0mg/kg body weight cured and the parasitaemia was not detected for more than 60 days. All mice in the control groups showed higher level of parasitaemia and died completely up to 4 days after infection. From this study it is indicated that the minimum curative dose of cymelarsan required to cure *Trypanosoma species* causing dourine (South Africa strain) in experimentally infected mice was greater than 1mg/kg. However, further studies should be conducted to know the effect of the drugs on the local strain of *Trypanosoma equiperdum* like Dodala strain from dourine endemic area in equine from Ethiopia so as to work out the best possible therapeutic strategies and/or alternative control measures.

Key words: Cymelarsan - Diminazene Diaceturate - Ethiopia, Mice - T. Equiperdum

INTRODUCTION

World equine population is about 111.2 million consisting of 44.5 million Donkeys, 57.6 million Horses and 5.02 Mules [1]. Among this, Ethiopia possesses 2.75 million horses, 5.02 million Donkeys and 0.63 million mules [2]. Horses have a prominent position in the agricultural and transport systems as draft, pack and riding animals. In a country where there is less developed modern transport and communication service, the natural choice rests on the let human and pack animals mode of transport, as it has been the case in some parts of the world. Thus, in a developing country like Ethiopia, the contribution of equines in the energy scenario is of considerable significance. The provisions of transport through pack animals, drawing carts, as riding animals or taxi operations, almost certainly contributes more to the national economy [3].

Despite the significance of horses in the sector of transportation and agriculture to the economy of the nation, the treatment accorded to these species of animal has been far below than the attention given to other economic animal species. This can partly be due to the age old erroneous concept that these species are hardy, tolerant and probably because their meat and milk is not edible in Ethiopia [4]. African horse sickness, Anthrax, Epizootic Lymphangitis, Dourine, Equine piroplasmosis, Horse mange, Rabies, Glander and Ulcerative Lymphangitis are among the major diseases affecting horses in Ethiopia [3].

Trypanosomosis is a serious parasitic disease, which occurs in large areas of Africa, Latin America, the Middle East and Asia. It affects most species of domestic livestock, many types of wild animals and human. The most important trypanosomes in terms of economic loss in domestic livestock are the tsetse transmitted species...
such as Trypanosoma congolense, Trypanosoma vivax and Trypanosoma brucei [5]. Mechanically transmitted trypanosomes such as Trypanosoma evansi and Trypanosoma vivax cause major production losses in the respective hosts.

Dourine is the only form of trypanosomosis caused by Trypanosoma equiperdum, which is transmitted directly from one animal host to another of the same species without the intervention of an insect vector but it transmitted almost exclusively during coitus. The causative agent of Dourine, T. equiperdum, differs from other mammalian trypanosomes also in the fact that it primarily tissue parasite. The clinical course of the disease depends on the pathogenicity of the trypanosome, the resistance of the breed and the physical condition of the animals. The incubation period ranges from two weeks till three months, clinical signs might be suppressed during cold months [6]. Generally the disease is divided in to three phases such as primary stage (Genital Oedema), secondary stage (plaques and skin eruptions) and tertiary stage (Neurological signs) [7]. Anemia, cachexia and genital oedema are often seen at post mortem [8]. Dourine is strictly limited to the equine. Thus the host range is limited to horses, donkeys and mules under natural condition [6].

Diagnosis of T. equiperdum, in horse, by standard parasitological techniques is difficult, owing to the low number of parasites present in the blood or tissue fluids and frequent absence of the clinical sign of the disease. Consequently the demonstration of trypanosomal antibodies in the serum has become the most important parameter in the determining the disease status of the animals [9]. The principal reason for using serological testes of diagnosis Trypanosomiasis is to over came the low level of sensitivity testes in detecting chronic infection. Alternative strategies, utilizing nucleic acid technologies such as the PCR also often high sensitivity and might be of use in the diagnosis of T. equiperpum [10].

For the treatment of T. equiperdum infection the same drug which is used for T. evansi are available. Evidence from in vitro drug sensitivity determination of T. equiperdum indicates that Suramin, Diminazen, Quinapyramine and Cymelarsan are effective against this trypanosome species [11, 12]. In Ethiopia, Dourine is an endemic problem of horses in the Arsi –Bale highland where treatment and control of the disease becomes difficult. The difficulty is mainly in terms of trypanocidal drug shortage which plays an influencing role in controlling and prevention of the disease in an endemic area [13, 14].

With the wide spread of drug resistant parasites in to all trypanosomosis endemic areas, development of new anti-trypanosomal compounds and drugs to circumvent resistance is urgently needed. However, it appears unlikely that new compounds will be introduced into the near future because of lack of interest by the pharmaceutical industry in investing in research and development of antitrypanosomal drugs [15]. Hence, there is an urgent need for assessment of efficacy of the drug in which representative number of trypanosomes isolated and examined for distribution and degree of efficacy so as to work out the best possible therapeutic strategies and/or alternative control measures. As prerequisite for such undertaking it is necessary to first obtain information on in vivo trypanocidal drug sensitivity patterns of the parasites in a mice model. Hence, the present study was initiated and designed to undertake studies related to the assessment of trypanocidal drug sensitivity of T. equiperdum OVI, South African strain with the available trypanocidal drugs. Therefore, the objective of the present study was to assess the trypanocidal drug sensitivity of Diminazene diaceturate and Cymelarsan against T. equiperdum OVI, South African strain in experimentally infected mice.

**MATERIALS AND METHODS**

**Study Area:** The present study was undertaken in molecular parasitology laboratory of Ethio-Belgium VLIR-UOS funded dourine project which is found inside the compound of Addis Ababa University Faculty of Veterinary Medicine, Debre Zeit, Ethiopia from November 2009 to April 2010. Debre Zeit is found 47Kms south east of Addis Ababa at (8° 44 N and 38° 58° E) at an altitude of 1850 meters above sea level. The mean annual rain fall is 885.4mm with a bimodal distribution. There are alternating dry and rainy season in the area. The long rainy extends from June to September and contributes about 84% of the total annual rainfall. While the dry season last from October to February. The short rainy season lasts from March to May. The mean annual minimum and maximum temperatures are 14°C and 26.3°C respectively with an overall average of 18.7°C. The mean relative humidity is 61.3 % [16].

**Experimental Animals:** Swiss white mice about 6 weeks of age weighing 20-25 gm were obtained from the breeding colony of the National Veterinary Institute (NVI). The mice were maintained under standard commercial pelleted ration and water ad libitum in the laboratory of the VLIR-UOS.
Sample Collection: Appropriate blood sample was collected regularly to evaluate the level of parasitemia by cutting the tip of the tail of the mice. Whenever large volume of blood (up to 2ml) is required for preparation of cryostabilates a cardiac puncture was performed after humanely euthanizing the mice with ether.

Experimental Design: Totally 60 mice were used in which 10 (grouped in to two having 5 mice each) for adaptation of the parasite and 50 for treatments and control. Initially, ten (10) donor mice (5 mice for each drug) were infected with T. equiperdum OVI South African strain stabilates originated from Institute of Tropical Medicine, Antwerp, Belgium. Before infected the cryostabilates were preserved in liquid nitrogen, following defrosting at room temperature or preferably at 37°C in water bath. The defrosted stabilates was then being mixed with Phosphate Substrate Glucose (PSG). The route of infection of the mice was intra peritoneal route with maximum dose of 0.2 ml.

The ten donor mice were checked for parasitemia daily by taking a drop of blood from the tip of the tail of the animals. Once parasitemia gets highly multiplied within three to five days, it was successfully possible to serially passage Trypanosoma equiperdum OVI South Africa strain to the next mice then mice were humanely euthanized to collect blood via intracardinal puncture. Each time the cryostabilates were prepared and kept in liquid nitrogen. Two drugs, diminazene diaceturate and Cymelarsan, were studied for their sensitivity on T. equiperdum OVI, South African strain. In each of the study 25 mice were divided randomly in to five experimental groups (group I-V) of five mice each. The first four groups (I-IV) comprise the treatment groups (those to be infected and treated with different doses of diminazene diaceturate). The fifth group (group V) served as infected untreated control group. While, the groups from VI to XV infected and treated with different doses of Cymelarsan, while group X kept as infected untreated control group. Mice in the treatment group were weighed on a digital balance prior to administration of the trypanocidal drugs.

The mice in the different treatment and control group were then being infected with T. equiperdum OVI, South African strain adapted to mice with 1-10 trypanosomes per preparation which is estimated to be $10^5 - 10^6$ trypanosomes per ml blood [17] by intraperitoneal inoculation with 0.2 ml blood from donor mice taken at peak parasitaemia.

Treatment and Monitoring: Diminazene diaceturate (Diminasan, Batch DG/20337 DUIPER WEG 9, 3449 JA woerden, Holland) and Bis (aminoethylthio) 4-melaminophenylarsine dihydrochloride (cymelarsan, lot B 09118A, MERIAL-17, rue Bourgelat 6900Z Lyon-France) were used for treatment of infected mice in the each treatment group. Diminazene diaceturate was administered via intraperitoneal route at doses of 3.5 mg/kg, 7.0 mg/kg, 14.0 mg/kg and 28.0 mg/kg body weight. Similarly Cymelarsan was administered via intraperitoneal route at doses of 0.25 mg/kg, 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg body weight (Table 1). The control groups received the same amount of sterile distilled water by intraperitoneal route in replacement of the treatment. Each drug was prepared by dissolving the required quantity of each compound in sterile distilled water before use according to respective manufacturer instruction and required dose of the drug was administered in 0.2 ml of solution for all treatment groups [18]. Mice were monitored every alternate day up to 60 days for the presence of trypanosomes by microscopic examination of wet smears of tail blood. Presence of trypanosomes in the blood smear was considered as indication of ineffectiveness of the trypanocidal drug used or possibly drug resistance.

Data Analysis: Data were collected, stored properly in Micro soft Excel sheet and then analyzed using GMP-5 Statistical soft ware [19].

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>No of mice per group</th>
<th>Diminazene Diaceturate (mg/kg bw)</th>
<th>Cymelarsan (mg/kg bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>7.0</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>14.0</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>28.0</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>5</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>VI</td>
<td>5</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>VII</td>
<td>5</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>VIII</td>
<td>5</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>IX</td>
<td>5</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>X</td>
<td>5</td>
<td>0*</td>
<td>0*</td>
</tr>
</tbody>
</table>

* Distilled water instead of Trypanocidal drug
Table 2: Results of diminazene diaceturate and cymelarsan to *Trypanosoma equiperdum* OVI South Africa strain in mice at different dose range.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Doses (mg/kg)</th>
<th>No. mice treated/relapsed</th>
<th>Mean relapse interval in days ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diminazene diaceturate</td>
<td>3.5 mg/kg</td>
<td>5/5</td>
<td>1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>7.0 mg/kg</td>
<td>5/5</td>
<td>1.2 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>14.0 mg/kg</td>
<td>5/5</td>
<td>1.4 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>28.0 mg/kg</td>
<td>5/4</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cymelarsan</td>
<td>0.25 mg/kg</td>
<td>5/5</td>
<td>6.6 ± 1.67</td>
</tr>
<tr>
<td></td>
<td>0.50 mg/kg</td>
<td>5/3</td>
<td>11.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>1.0 mg/kg</td>
<td>5/0</td>
<td>Cured</td>
</tr>
<tr>
<td></td>
<td>2.0 mg/kg</td>
<td>5/0</td>
<td>Cured</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Distilled water</td>
<td>10/10</td>
<td>Died after 3 days of post infection but after one day of parasitemia detection.</td>
</tr>
</tbody>
</table>

* = Treated / Died

**RESULTS**

Diminazene diaceturate at doses 3.5mg/kg, 7mg/kg and 14mg/kg body weight failed completely to cure but only 1(20%) mice was survived up to 60 days. Which treated at dose of 28mg/kg body weight (Table 2). However, after 60 days of dexamethasone inoculation, parasitemia of dourine causing *Trypanosoma spp* (South Africa strain) was demonstrated in the blood of the mouse. In case of cymelarsan, mice infected with *T. equiperdum* OVI South Africa strain and treated at dose of 0.25mg/kg body weight were were completely cured but 2(40%) of mice treated at a dose of 0.50mg/kg body weight were relapsed within 11.0 ± 1.0 days. However, mice treated with cymelarsan at higher doses of 1.0mg/kg and 2.0mg/kg body weight were completely cured from the infection with no relapse at least for more than 60 days. All mice in the control groups showed higher level of parasitaemia and died completely up to 4 days after infection. Hence, as per this study the minimum curative dose (MDC) of cymelarsan required to cure *Trypanosoma equiperdum* OVI from experimentally infected mice was recorded to be more than 1mg/kg.

**DISCUSSION**

Blood from infected horses produce disease very rarely when inoculated directly in to healthy mice. Therefore, in the current study the infection was first established in mouse using already prepared cryostablates of the parasite from horse and then it was inoculated in to study mice groups. Parasite appeared in the blood of study mice with in 2 or 3 days and death occurred after a heavy parasitemia development which may last only 3 to 5 days after infection [20].

The results from the drug sensitivity showed that Diminazene diaceturate at dose range of 3.5mg/kg, 7.0mg/kg, 14mg/kg body weight failed completely to cure *Trypanosoma equiperdum* OVI South Africa strain due to sever parasitemia. However, only 1(20%) mouse was survived up to 60 days after treatment with diminazene diaceturate at dose of 28mg/kg body weight. The mean relapse interval for the dose range of 3.5mg/kg, 7.0mg/kg, 14.0mg/kg and 28mg/kg body weight was 1± 0.0, 1.2 ± 0.45, 1.4 ±0.55 and 2.5 ± 1.0 days respectively.

From other similar studies it was indicated that diminazene diaceturate is less effective against Trypanosomes of subgenus Trypanozoon than *T. congolense* and *T. vivax*. This could be attributed to the relatively rapid excretion of the drug [5]. Moreover, diminazene diaceturate can not cross the blood brain barrier and somatic tissue. Due to this fact, it cannot be the curative drug for trypanosomes with tissue affinity such as *T. equiperdum* [11, 12]. The relapse of *T. equiperdum* after 60 days post diminazene diaceturate therapy could be associated with the release of the trypanosomes from tissues inaccessible by the drug.

Although diminazene diaceturate probably exerts its action at the level of the kinetoplast DNA, this has not been proven *in vivo* and other mechanisms of action cannot be excluded [21]. Similarly the molecular basis of resistance to diminazene in trypanosomes is not clear. The accumulation of diminazene was markedly reduced in arsenical-resistant *T. brucei brucei* owing to alterations in the nucleoside transporter system. However, there might be other resistance mechanisms [11].
The result from the drug sensitivity study showed that only a single shoot of cymelarsan at dose of 1.0mg/kg and 2.0 mg/kg body weight becomes effective treatment for Trypanosoma equiperdum OVI South Africa strain in experimentally infected mice with no relapse for at least two months. However, cymelarsan at doses rate of 0.25mg/kg body weight failed to cure the infection but in case of 0.5mg/kg only 2(40%) mice survived. In lower doses of cymelarsan at 0.25mg/kg and 0.5mg/kg body weight mean relapse intervals were between 6.6 ± 1.67 and 11.0 ±1.0 days.

Cymelarsan however, was found to be ineffective against Trypanosoma equiperdum in mice at doses of 0.25mg/kg and 0.5mg/kg body weight. The inability of this drug to clear parasitemia in mice as well as domestic animal at the standard dose of 0.25mg/kg for T.evansi has supported by previously reports. For instance cymelarsan was ineffective in buffaloes treated at doses ranging from 0.5mg/kg to 3mg/kg [22], in goats treated at a dose of 0.3mg/kg [23], in mice treated at dose of 0.25 and 0.5mg/kg [24] and in cattle treated at a dose of 0.5mg/kg [25].

Cymelarsan was found to be very effective against T. equiperdum, T. evansi and T. brucei brucei, in horses, camels, buffalo, goats and pigs [11, 22, 23, 26]. The arsenical compound contains the trivalent arsenic element with a markedly reactive arsenoxide group. The presence of arsenoxide confers the physicochemical ability of lipid solubility that allows passage across the blood brain barrier (BBB) [27]. The arsenical compound melarsoprol revealed the remarkable ability to cross the BBB and kill Trypanosoma brucei gambiensce and Trypanosoma brucei rhodesiense parasites residing in the CSF.

It was clearly evident that cymelarsan was quite effective against T.evansi infection in camels at standard recommended dose rate of 0.25mg/kg body weight [28]. This ineffectiveness of the drug for the present study probably confirms the suggestion made previously [23] that the recommended dose might have been applied strictly for the treatment of camels only but the higher doses are needed to treat T. evansi in other animals and mice. By the same scenario in the present study the mice were treated under dosage where the metabolic weight of camels versus mice should be considered.

The proposed mechanism of action of the drug can be explained in terms of the very strong binding characteristics of cymelarsan with targets on the parasite known as thiol containing enzymes like glycerol-3-phosphate dehydrogenase (G3PDH) [29].

Hence, it can be recommended to the OIE to replace the current strategy of eradication by an appropriate drug treatment. Yet, currently stamping out strategy is applied by the OIE with slaughtering of CFT positive horses where treatment is prohibited [30]. However, it is not economically feasible to apply strict test and slaughter policy of the OIE to control dourine at least in developing country like Ethiopia. From the present study mice infected with T.equiperdum OVI South Africa strain were cure after being treated with cymelarsan than those treated with diminazene diaceturate. Therefore, cymelarsan can be recommended as choose of treatment in controlling strategy of the studied Trypanosomes species.

**CONCLUSION**

Trypanocidal drugs remain the principal method of animal Trypanosomiasis control in most African counties. However, there is growing concern that future effectiveness may be severely curtailed by wide spread drug resistance. The present study was done in accordance with the aim of assessing the Trypanocidal drug efficacy of diminazene diaceturate and cymelarsan against T.equiperdum OVI South Africa strain in experimental infected mice. As study result indicate that cymelarsan at dose rate of 1mg/kg and above was found highly effective than diminazene diaceturate considering the toxicity of the drug if over dosed. Regarding animal welfare this would be a big step forward, because it can release an alarming sign to the developed countries that apply the OIE’s test and slaughter policy. To combat the problems associated with trypanosomes, further research into existing drugs is a prerequisite for their optimal usage in the overall effort of improving animal health and productivity through control of trypanosomiasis.

Therefore, in vitro assays should be performed to detect drug resistance against the available isolates of Trypanosoma equiperdum from equine rearing area of Ethiopia where dourine is endemic and major problem. The current strategies of OIE against dourine eradication programmes should be reconsidered, since there could be some effective drugs against Trypanosoma equiperdum. Besides, The toxicity of the cymelarsan should further be studied in Ethiopia before being imported and distributed it in endemic area to control dourine.

**ACKNOWLEDGEMENTS**

This investigation received financial support from Ethio-Belgium VLIR-UOS funded dourine project and free access of laboratory facilities, technical and kind support from Addis Ababa University College of Veterinary
Medicine. The authors also extend special acknowledgement to Dr. Fufa Dawo, Dr. Yacob Hailu and Mr. Alemu senior laboratory technician for their professional support.

REFERENCES


